Aus der Medizinischen Klinik mit Schwerpunkt Nephrologie
der Medizinischen Fakultät der Charité–Universitätsmedizin Berlin

DISSERTATION

Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy

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ABBREVIATIONS

A	Ampere
ACE	Angiotensin-converting enzyme
AECA	anti-endothelial cell antibodies
Ang II	Angiotensin II
AMR	Antibody-mediated rejection
AP-1	activator protein 1
APS	ammonium persulfate
AT_1R-Ab	AT_1R-activating antibody
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
BSA	bovine serum albumin
bp	base pair
B_2	Bradykinin
B_2M	Beta-2-Microglobulin
B_2R	Bradykinin B_2 receptor
CB_1R	Cannabinoid receptor Type I
CAV	cardiac allograft vasculopathy
cDNA	complementary DNA
cm	centimeter
Conc.	Concentration
Co-IP	Co-immunoprecipitation
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
ECE	Endothelin-converting enzyme
ET_AR	Endothelin-1 type A receptor
ET_BR	Endothelin-1 type B receptor
ET_AR-Ab	ET_AR-activating antibody
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
ET-1	Endothelin 1

ET-2	Endothelin-2
ET-3	Endothelin-3
FRET	fluorescence resonance energy transfer
FBS	fetal bovine serum
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptors
HEK293 cells	Human Embryonic Kidney cells
HMECs	Microvascular endothelial cells
HLA	human leukocyte antigen
h	hour
Ig	immunoglobulins
IgG	immunoglobulin class G
IL	Interleukin
IP	immunoprecipitation
IL-8	Interleukin-8
M	molar
min	minute
mm	millimeters
mL	milliliter
mg	milligram
MHC	major histocompatibility complex
MOPS	3-(N-morpholino)-propanesulfonic acid
mRNA	Mitochondrial RNA
mL	microliter
mM	micromolar
mg	microgram
L	Liter
nM	nanomolar
kD	Kilodalton
NF- κB	Nuclear factor-κB
p	phospho
PAH	pulmonary arterial hypertension

PBS	Dulbecco’s Phosphate Buffered Saline
PCR	polymerase chain reaction
P-IgG	Positive IgG fraction
P/S	Penicillin/Streptomycin
qPCR	quantitative real time polymerase chain reaction
RAAS	renin-angiotensin-aldosterone system
RT	room temperature
rpm	revolutions per minute
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
s	second
SD	standard deviation
SSc	systemic sclerosis
TEMED	Tetramethylethylenediamine
Temp.	temperature
TGFβ1	growth factor β gene 1
TF	tissue factor
µL	microliter
V	voltage
VADS	ventricular assist devices
VCAM-1	vascular cell adhesion molecule-1
x g	times gravity

Abstract

Background:

Autoantibodies simultaneously targeting two G protein-coupled receptors, Angiotensin II type 1 (AT_1R) and Endothelin-1 type A receptor (ET_AR), induce severe obliterative vasculopathy observed in graft rejection and systemic sclerosis. A molecular toolbox was generated to study the cross-talk between AT_1R and ET_AR in the context of obliterative vasculopathy.

Methods:

HEK cell lines over-expressing stably the tagged receptors individually or dually were generated by transfection and appropriate antibiotics selection. The clones over-expressing the tagged receptors were selected and verified by western blot and immunoprecipitation. IgG were isolated from patients with obliterative vasculopathy by HiTrap Protein G columns, and $F(ab)_2$ fragments without Fc were generated by pepsin digestion. A GPCR activation assay was elaborated by expressing isolated human GPCRs in genetically modified yeasts.

Results:

HEK cell lines over-expressing stably individually and dually tagged AT_1R and ET_AR were successfully generated. The over-expressed receptors triggered ERK phosphorylation both in endogenous and immune context. To verify the role of autoantibodies in the receptors immune-mediated activation, $F(ab)_2$ fragments without Fc were generated. In the dual stable cell, $F(ab)_2$ fragments were significantly biologically active ($p<0.05$). The stable cell lines were used to study the interaction between the AT_1 and ET_A receptors. After having verified that antibodies directed against the tagged receptors specifically immunoprecipitated them, co-immunoprecipitation experiments were performed. The results suggest that the receptors could interact. As the second extracellular loop of the receptors could be involved in their immune activation, ECL2 of AT_1R and ET_AR were mutated. A GPCR activation assay was elaborated to assess the activation of isolated human receptors. The results showed that endogenous and immune-mediated activation of the receptors depend on the ECL2 in AT_1R but not in ET_AR.

Conclusion:

A molecular toolbox based on stable human cell lines and a yeast GPCR activation assay has been elaborated. It was used to gain knowledge on the cross-talk between the Angiotensin II type 1 and the Endothelin-1 type A receptors. The results suggest that AT_1R and ET_AR might form constitutive heterodimers. Besides, IgG digestion brought convincing evidence that antibodies

against AT_1R and ET_AR play an important role in transducing intracellular signals. The results also confirmed that the second extracellular loop is a conformation-sensitive site for natural ligand and autoantibodies in AT_1R but not in ET_AR. This molecular toolbox is thus of great advantage in understanding the pathogenesis of immune-mediated obliterative vasculopathy and discovering new therapeutic strategies.

Zusammenfassung

Hintergrund:

Autoantikörper, die gleichzeitig gegen zwei G-Protein gekoppelte Rezeptoren, den Angiotensin II Typ 1 Rezeptor (AT_1R) und Endothelin-1 Typ A Rezeptor (ET_AR) gerichtet sind, sind Auslöser schwerer obliterativer Vaskulopathien, wie Sie bei Transplantatabstossung und Systemischer Sklerose beobachtet werden. Es wurden molekulare Modelle generiert, die ein Studium des Crosstalks zwischen AT_1R und ET_AR in diesem klinischen Kontext ermöglichen.

Methoden:

Durch Transfektion und entsprechende Antibiotikaselektion wurden HEK Zelllinien generiert, die individuell oder dual getaggte Rezeptoren stabil exprimierten. Die getaggte Rezeptoren überexprimierenden Klone wurden selektiert und durch Westernblots und Immun-präzipitationen verifiziert. Mittels HiTrap Protein G Säulen wurde IgG von Patienten mit obliterativer Vaskulpathie isoliert und $F(ab)_2$ Fragmente ohne Fc durch Pepsinverdau generiert. Zur Testung der GPCR Aktivierung wurde ein Assay ausgearbeitet mit genetisch veränderte Hefen, die isolierte humane GPCR exprimieren.

Ergebnisse:

HEK-Zelllinien, die stabil individuell oder dual getaggte Rezeptoren AT_1R und ET_AR exprimieren wurden erfolgreich generiert. Die überexprimierenden Rezeptoren triggerten ERK Phosphorylierung sowohl im endogenen, als auch immunen Kontext. Zur Verifizierung der Rolle der Autoantikörper in der immun-vermittelten Aktivierung, wurden $F(ab)_2$ Fragmente ohne Fc generiert. In der dual stabilen Zelllinie, waren die $F(ab)_2$ Fragmente signifikant biologisch aktiv ($p<0.05$). Die stabilen Zelllinien wurden zur Untersuchung der Interaktion zwischen dem AT_1R und ET_AR verwendet. Mit den gegen die getaggten Rezeptoren gerichteten Antikörper wurden Koimmunpräzipitationen durchgeführt. Die Ergebnisse wiesen auf eine Interaktion zwischen den beiden Rezeptoren hin. Da der zweite extrazelluläre Loop der Rezeptoren bei ihrer Immunaktivierung beteiligt sein könnte, wurden der ECL2 des AT_1R und ET_AR mutiert. Zur Testung der GPCR Aktivierung wurde ein Assay zur Aktivitätsbestimmung isolierter humaner GPCR ausgearbeitet. Damit konnte gezeigt werden, dass endogene und immunvermittelte Aktivierung der Rezeptoren durch den ECL2 nur beim AT_1R, nicht jedoch beim ET_AR bestimmt wird.

Schlussfolgerung:

In der vorliegenden Arbeit wurden molekulare Werkzeuge mit stabilen humanen Zelllinien und ein GPCR-Aktivitätsassay in einem Hefemodell erstellt. Diese wurden benutzt, um Kenntnisse

über den Crosstalk zwischen Angiotensin II Typ 1 und Endothelin-1 Typ A Rezeptor zu erzielen. Die Ergebnisse legen es nahe, dass AT_1R und ET_AR konstitutive Heterodimere ausbilden könnten. Darüberhinaus zeigte IgG Verdau auf, dass Antikörper gegen AT_1R und ET_AR eine wichtige Rolle in der Vermittlung intrazellulärer Signale spielen. Die Ergebnisse bestätigten auch, dass der zweite extrazelluläre Loop eine konformationssensitive Stelle für den natürlichen Liganden und Autoantikörper bei AT_1R, nicht jedoch ET_AR darstellt. Diese molekularen Werkzeuge sind daher ein großer Gewinn, die Pathogenese immunvermittelter obliterativer Vaskulopathien zu verstehen und neue therapeutische Strategien aufzudecken.

1 Introduction

1.1 Obliterative vasculopathy

1.1.1 Clinical manifestations

Obliterative vasculopathy represents pathological changes occurring in different diseases such as thromboangiitis obliterans (TAO) or Buerger's disease,[1] preeclampsia,[2] transplant rejection[3] and systemic sclerosis (SSc).[4] As an example, the obliterative transplant vasculopathy is a complication in human allografts, typically heart and kidney, which can lead to renal-allograft rejection, chronic cardiac allograft rejection, and restricts the time of organ survival considerably[5]. Obliterative vasculopathy is characterized by vascular occlusion leading to compromised blood supply[6] (Figure 1). It can affect all types of blood vessels including arteries, veins, and capillaries, and its specific symptoms and signs depend on which organs have been damaged and the extent of the damage[7,8]. In obliterative transplant vasculopathy, vascular lesions affect predominantly the afferent arteries and the arterioles leading to allograft loss after organ transplantation and allograft failure[9]. In diffuse SSc, the widespread obliterative vasculopathy affects small arteries and capillaries leading to multi-organ systemic disorder[4]. Obliterative vasculopathy can be triggered by autoimmune disorder which occurs when the immune system produces antibodies that attack and damage the body's own tissues or cells[10,11,12].

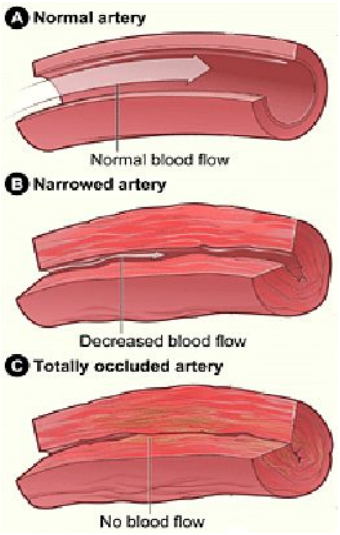


Figure1. Progression of obliterative vasculopathy. (A) normal artery. (B) narrowed artery with decreased blood flow. (C) occluded artery and scarring on the artery wall.

1.1.2 Pathogenesis of obliterative vasculopathy in immune-mediated diseases

1.1.2.1 Obliterative transplant vasculopathy

Transplantation has emerged as a viable therapeutic strategy for patients with end-stage organ disease, offering extended survival and improved quality of life. Chronic rejection is the single most significant obstacle to long term organ allograft survival[13]. Chronic rejection usually has an insidious onset and is characterized primarily by obliterative arteriopathy, interstitial fibrosis and atrophy of parenchymal elements. Obliterative arteriopathy is the pathognomomic for chronic rejection in all vascularized solid organ allografts[14].

Obliterative arteriopathy in transplantation evolves from a repair response to immune-mediated vessel wall injury, and all three layers of the artery wall are involved: intima, media and adventitia[14]. In intima, early findings are endothelial activation and damage leading to the disruption of endothelial homeostasis[15]. In the media, early changes include edema, and degeneration or frank necrosis of individual myocytes[16]. Early after transplantation, the adventitia is a primary site of sensitization, which results in inflammation and edema[17]. Ultimately, disruption of arterial wall homeostasis and luminal narrowing predisposes to thrombosis and organ ischemia[14]. Prototypic events are reconstructed in Figure 2.

The obliterative vascular changes associated with rejection were first described in 1964[3]. Its pathogenesis probably involves both humoral and cell-mediated responses by the recipient to specific antigens present in the donor tissue[18,19]. However, this does not account for graft rejection occurring in organs without Human Leukocyte Antigens (HLA) mismatches. Dragun et al.[10] suggested that non-HLA non-complement-fixing agonistic antibodies reacting to artery-specific antigens could be responsible for the severe vascular injury.

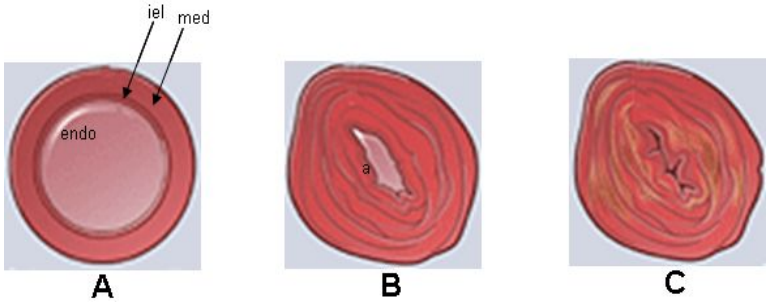


Figure 2. Schematic cross-section of an artery showing the sequential steps of obliterative arteriopathy. (A) anatomic features in a normal artery including the endothelial lining (endo), the internal elastic lamina (iel) and the media (med). (B) early arterial changes during rejection. (C) later changes.

1.1.2.2 Obliterative vasculopathy in systemic sclerosis

Systemic sclerosis is a chronic multisystem autoimmune disease with a widespread obliterative vasculopathy of small arteries that is associated with varying degrees of tissue fibrosis and with numerous cellular and humoral immunological abnormalities[20]. The most common first symptom

of scleroderma is Raynaud's phenomenon[21]. As the disease progresses, the vasculopathy proceeds to significant organ manifestations, including renal crisis and pulmonary arterial hypertension (PAH), contributing significantly to morbidity and mortality, which share similarities in their underlying vasculopathy[22]. It is well established that endothelial cell dysfunction is a key event in SSc pathogenesis appearing early in the course of the disease and preceding fibrosis[1].

Obliterative vascular lesions in SSc resemble those in transplant vasculopathy[12]. Vasculopathy involves all layers of the vessel wall, and is characterized by fibrotic intimal hyperplasia[23]. Occlusion of the small arteries can facilitate the formation of in situ-thrombosis, affecting any organ or any part of the body and resulting in Raynaud's phenomenon, PAH, and renal involvement[24] (Figure 3). Although the etiology of injury mechanisms has not yet been fully elucidated, a growing body of evidence suggests that autoantibody-mediated receptor stimulation contribute to disease pathogenesis linking the major pathophysiological features of SSc[25].

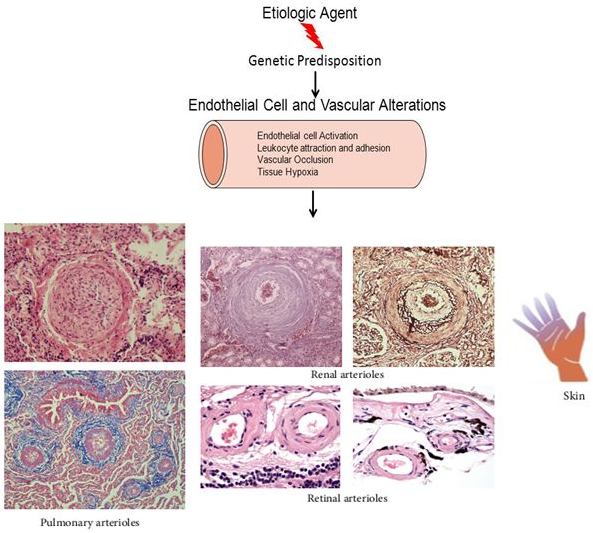


Figure 3. Overall scheme illustrating vasculopathy in SSc[24]. An unknown causative agent induces activation of immune and inflammatory cells resulting in endothelial cell damage. The release of cytokines and growth factors by endothelial cells, myofibroblasts and inflammatory cells can disrupt the intimal homeostasis. The vascular alterations progress to multiple internal organs. Later stages of the disease are marked by increasingly avascular areas. Despite the obliteration of microvascular structures, compensatory vasculogenesis and angiogenesis do not occur normally.

1.2 Vasoactive peptides in obliterative vasculopathy

Numerous vasoactive peptides play an important role in regulating vascular tone, reactivity and structure[26]. In pathological conditions, alterations in the regulation of vasoactive peptides result in endothelial dysfunction, vascular remodeling and vascular inflammation, which are important processes underlying obliterative vasculopathy[26]. For instance, serotonin (5-hydroxytryptamine) and Angiopoietin 1 (Ang-1) are involved in severe pulmonary hypertension leading to diffuse

medial thickening in small pulmonary vessels and eventually resulting in obliteration of the distal pulmonary arterial tree[27]. Angiotensin II (Ang II), Endothelin-1 (ET-1) are primarily vasoconstrictors with growth-promoting actions and are particularly significant because of their pleiotropic actions[26]. Both have been identified in many forms of obliterative vasculopathy[12,11,28].

1.2.1 Angiotensin II

1.2.1.1 Biosynthesis of Angiotensin II

Angiotensin II is a bioactive peptide of the renin-angiotensin system which is considered to play an important role of regulating blood pressure, electrolyte balance and it has renal, neuronal as well as endocrine functions related to cardiovascular control[29]. Ang II is the resulting product of a cascade of enzymatic reactions, starting with the substrate angiotensinogen which is converted by renin to angiotensin I and further converted to Ang II by the angiotensin-converting enzyme (ACE)[30] (Figure 4).

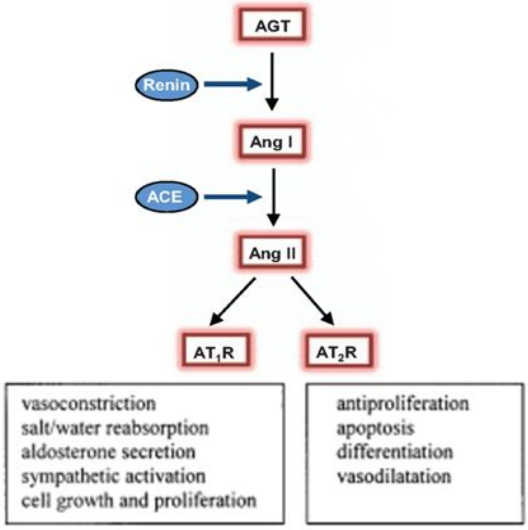


Figure 4. Generation of biologically-active angiotensin[31].

1.2.1.2 Angiotensin II in obliterative vasculopathy

Angiotensin II is a multifunctional peptide that has numerous actions in vascular physiology and pathology[32]. Although the principal function of Ang II is vasoconstriction, it has become evident that Ang II has important properties for regulating cell growth and apoptosis, influencing cell migration and extracellular matrix deposition[33]. Accordingly, Ang II plays a fundamental role in controlling the functional and structural integrity of the arterial wall and may be important in pathophysiological processes regulating hypertrophy and hyperplasia of vascular smooth muscle cells and vascular remodeling in various obliterative vasculopathy diseases. For example, Ang II

contributes to the arterial remodeling, atherosclerosis and restenosis which accompanies pulmonary hypertension[34].

1.2.2 Endothelin-1

1.2.2.1 Biosynthesis of Endothelin-1

The endothelin family consists of three isoforms: Endothelin-1, Endothelin 2 (ET-2), and Endothelin 3 (ET-3)[35]. ET-1 was first isolated in 1988 and identified as an endothelial cell-derived vasoconstrictor peptide, and it is the principal isoform of the endothelin peptide family[36]. The human gene for ET-1 is located on chromosome 6 and encodes a 21-amino acid peptide. ET-1 is generated from precursor molecule called preproET, and constituted by preproET-1 peptides, two activating peptidases and two endothelin receptors, ET_AR and ET_BR[37] (Figure 5).

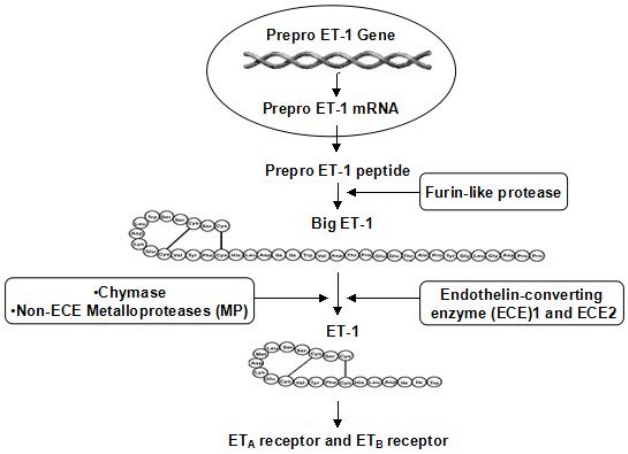


Figure 5. Schematic representation of Endothelin-1biosynthesis[38].

1.2.2.2 Involvement of Endothelin-1 in obliterative vasculopathy

Endothelin-1 is a potent vasoconstrictor peptide originally isolated from endothelial cells[39]. It is responsible for a variety of cellular events: contraction, proliferation, apoptosis in an array of physiological processes such as embryonic development, reproduction, angiogenesis, and cardiovascular homeostasis[40]. It has been reported that in addition to its systemic hypertensive effects, ET-1 contributes to the development of vascular diseases by having a localized effect triggering inflammation and oxidative stress, increasing growth factors and proliferative factors levels, and leading to the production of collagen and extracellular matrix[41]. Accordingly, ET-1 plays a key role in the pathophysiology of various obliterative vasculopathy diseases such as pulmonary hypertension and vascular remodeling. Becker et al[28]. suggested that vasoconstrictive and vascular remodeling actions of ET-1 are involved in the pathogenesis of PAH.

1.3 G protein-coupled receptors for Angiotensin II and Endothelin-1

G protein-coupled receptors (GPCRs) are transmembrane protein receptors that contain seven transmembrane α-helical regions and bind to a wide range of ligands[42]. GPCRs perceive many extracellular signals and transduce them to heterotrimeric G proteins, which further activate appropriate downstream intracellular effectors and thereby play an important role in various signaling pathways[43]. Typical of GPCRs, activation of Angiotensin II and Endothelin-1 receptors initiate several processes[44].

1.3.1 Angiotensin II receptors

Angiotensin II is the endogenous ligand for the Angiotensin II type 1 and 2 receptors, AT_1R and AT_2R, respectively[45]. In humans, AT_1R is widely expressed at relatively constant levels in adults and is localized in numerous tissues, including kidney, heart and vasculature[45]. In contrast, the AT_2 receptor is mainly present during fetal development. AT_1R mediates most of the well-known effects of Ang II, which include blood pressure regulation and osmoregulation[46]. Ang II acts also via AT_1R in cell growth, proliferation, fibrosis and inflammation of vascular smooth muscle and endothelial cells leading to vascular pathologies such as endothelial dysfunction[47,48]. Effects of AT_2R are less clear. It has been proved to be associated with the control of cell proliferation and differentiation, angiogenesis and tissue regeneration, as well as apoptosis[49]. Some studies proved that chronic AT_2R activation attenuates AT_1R function and blood pressure and suggested that AT_2R appears to have functions opposite to or balancing those of AT_1R[50].

1.3.1.1 Structure of the Angiotensin II type 1 receptor

The human genome contains a unique gene coding for AT_1R, which is localized on chromosome 3. The amino acid sequence consists of 359 amino acid residues with a calculated molecular weight of 41 kDa[51]. Structural predictions suggest an extracellular NH_2-terminus followed by seven α-helical transmembrane spanning domains, which are connected by three extracellular (ECL) and three intracellular loops, linked to the carboxyl-terminus[51] (Figure 6). Two pairs of disulfide bridges have been identified between four cysteine (Cys) residues located in the extracellular domain[52]. One of these bridges, spanning the transmembrane domain 3 and the second ECL (Cys 101 and Cys 180), is highly conserved in AT_1R[52]. The second disulfide bridge connects the N-terminus and third ECL between Cys 18 and Cys 274[51].

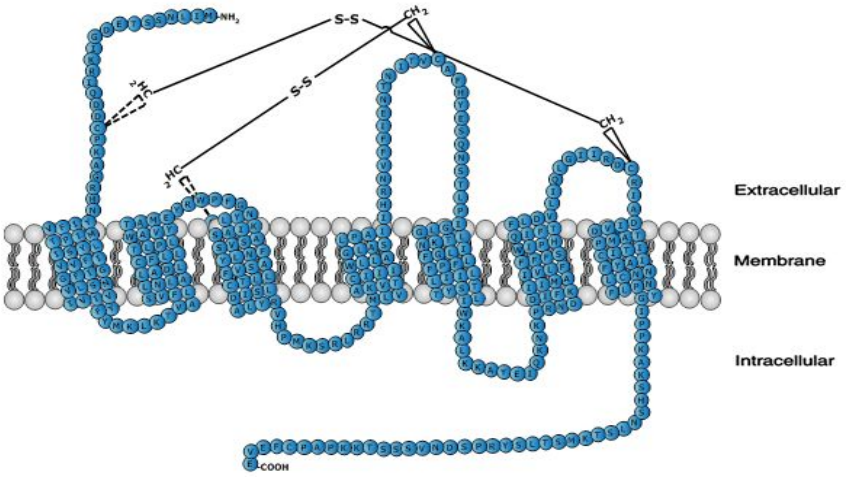


Figure 6. Structure of the Angiotensin II type 1 receptor.

1.3.1.2 Interaction between AT_1R and other GPCRs

AT_1R undergoes agonist-induced dimerization, including homo- or hetero-dimerization[53]. Some studies indicate that AT_1R can exist as homodimers in transfected COS-7 cells co-expressing wild type AT_1R and AT_1R mutants[54]. The homodimerization of wild type and mutant AT_1R can inhibit G protein (Gαq) signaling, whereas ERK phosphorylation and β-arrestin recruitment stay unaffected, suggesting that the AT_1R oligomerization has differential effects on signaling pathways[54]. Angiotensin II type 1 receptor can also interact with its counterpart, AT_2R. *In vitro*, it has been observed that AT_2R binds directly to the AT_1R and antagonizes it[55]. *In vivo*, the heterodimerization of the AT_2R and AT_1R was detected on human myometrial biopsies[56]. Besides, interaction of AT_1R with other GPCRs has been studied. Bradykinin (B_2), a vasodepressor, is a functional antagonist of Ang II[57]. Abdalla et al.[58] reported that AT_1/B_2 receptors heterodimerized *in vitro*, induced activation of G proteins and enhanced AT_1R signaling in smooth muscle cells. Furthermore, they found that AT_1/B_2 receptor heterodimers increased ET-1 secretion of mesangial cells in hypertensive rats[58]. Nevertheless, this interaction is still controversial[59]. Zeng and colleagues reported in many papers that AT_1R can form heterodimers with Endothelin-1 type B receptor (ET_BR). They speculated that this might be important for the development of hypertension *in vivo*[60,61].

1.3.2 Endothelin-1 receptors

Endothelin-1 binds to two GPCRs, Endothelin-1 type A (ET_AR) and B (ET_BR) receptors. Generally, ET_AR is considered to be the primary vasoconstrictor and growth-promoting receptor[62]. The binding of endothelin-1 to ET_AR increases vasoconstriction and the retention of sodium, leading to increased blood pressure[62]. ET_BR inhibits cell growth and vasoconstriction in

the vascular system[63,64]. ET_AR and ET_BR are distinctive in their localization[65]. ET_AR can be found in many human tissues including vascular smooth muscle, heart, lung and kidney while expression in the endothelium does not exist[65]. ET_BR is primarily localized in the brain but can be found in the aorta, heart, lung and kidney[65]. Activation of ET_AR in vascular smooth muscle cells mediates vasoconstriction and cell proliferation, whereas activation of ET_BR on endothelial cells leads mainly to vasodilatation, and regulates the synthesis of ET-1[66]. Interestingly, ET_BR on smooth muscle cells can elicit vessel contraction[65]. Balanced activation of the two receptors maintains vascular tone and regulates endothelial cell proliferation, whereas imbalance in this system contributes to the onset of hemodynamic disorders[67].

1.3.2.1 Structure of the Endothelin-1 type A receptor

Human ET_AR is 427 amino acids long and encoded by a gene located on chromosome 4[68]. Immunoblot analysis of vascular tissues has shown an intense band with an apparent molecular mass of 59 kDa, and less dense bands at 44 and 32 kDa[69]. ET_AR is composed of a long N-terminus, seven helical transmembrane domains, three extracellular and three intracellular loops, and an intracellular C-terminal tail (Figure 7). One disulfide bridge has been identified, spanning the transmembrane domain 3 and the second ECL (Cys 158 and Cys 239).

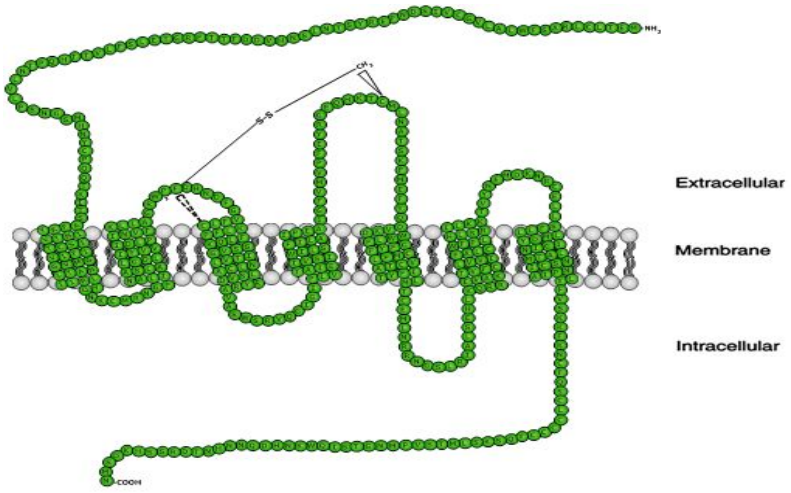


Figure 7. Structure of the Endothelin-1 type A receptor

1.3.2.2 Interaction between ET_AR and other GPCRs

The presence of homo- and heterodimers of the endothelin receptors has been recently observed *in vitro* and *in vivo* and it possibly accounts for the complexity of ET-1 responses[70]. ET_AR can form homodimers or heterodimers with ET_BR but the functional significance is not clear[71]. *In vitro*, Gregan et al.[72,73] generated plasmid-encoding fusion proteins of ET_AR and ET_BR with different tag and fluorochromes, and showed that ET_AR exist as homodimers and ET_AR and

ET_BR form heterodimers. In pulmonary arteries of rats, Sauvageau et al.[74] also demonstrated a heterodimerization of the two endothelin receptors. Dimerization of receptors alters their ligand-binding properties and in some cases appears essential for activation of downstream signaling in certain cell types[75]. The homo-dimers of ET_AR and ET_AR or ET_BR and ET_BR are characterized by different binding density[76]. Moreover, the functional response is different, depending on whether homo- or heterodimers are formed: the binding of ET-1 to homodimers induces a transient elevation in Ca^{2+} concentration, while the response mediated by heterodimers lasts for several minutes[77]. For endothelin receptors, most work to date has focused on heterodimerization of ET_AR and ET_BR, rather than heterodimerization of endothelin receptors with receptors that bind other ligands[64]. Hamilton et al.[78] reported that ET_AR can form dimers with α_1-adrenoceptor other receptors and ET_AR activation may impair α_1-adrenoceptor-mediated responses. It may provide evidence for endothelin binding sites and functional studies.

1.4 Role of agonistic antibodies in immune-mediated obliterative vasculopathy diseases

Vascular obstruction and endothelial cell dysfunction play a central role in diseases such as transplant vasculopathy and SSc, characterized by the activation of the immune system triggering the production of autoantibodies[10,12]. Humoral immune response is primarily directed against antigens expressed on endothelial cells and epithelial cells and categorized as non-HLA alloantigens or tissue-specific autoantigens[79]. Many clinical studies have examined the clinical relevance of non-HLA antigens in solid organ transplantation and provided an update on non-HLA-related humoral immunity in the autoimmune diseases[80,81]. It has been reported that agonistic autoantibodies directed against the Angiotensin II type 1 receptor (AT_1R-Abs) and Endothelin-1 type A receptor (ET_AR-Abs) are involved in the pathophysiology of autoimmune vasculopathy in transplantation and SSc[12,82].

1.4.1 AT_1R-Abs and ET_AR-Abs in transplantation

Antibodies against AT_1R and ET_AR have been reported in several transplant vascular pathologies[11,12,82]. Elevated levels of AT_1R- and ET_AR-activating antibodies have been associated with antibody-mediated rejection (AMR) in the absence of donor HLA specific antibodies in renal transplantation[82] and heart transplantation[11].

1.4.1.1 AT_1R-Abs and ET_AR-Abs in kidney transplantation

Anti-AT_1R antibodies have been shown to be directly involved in the pathophysiology of immune-mediated vascular diseases such as preeclampsia and malignant hypertension[83,84,85,86]. Dragun et al.[10] investigated the potential role of anti-AT_1R antibodies in kidney-transplant recipients with steroid-refractory acute renal-allograft rejection who did not have anti-HLA antibodies. *In vitro* stimulation of vascular cells with AT_1R-Abs induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and increased the DNA binding activity of the transcription factors Activator Protein 1 (AP-1) and Nuclear Factor-κB (NF- κB)[10] (Figure 8). The authors also found that passive antibody transfer into rats, which underwent kidney transplantation, induced vasculopathy and hypertension, indicating that AT_1R-Abs play an active role in the pathophysiology of the disease[10]. Furthermore, effects of AT_1R-Abs were blocked by the AT_1R antagonist, losartan *in vitro*[10] and *in vivo*[87]. In addition, patients originally identified with AT_1R-Abs were treated with a combination therapy (plasmapheresis, intravenous immunoglobulin infusions and losartan) which improved renal function and graft survival[10].

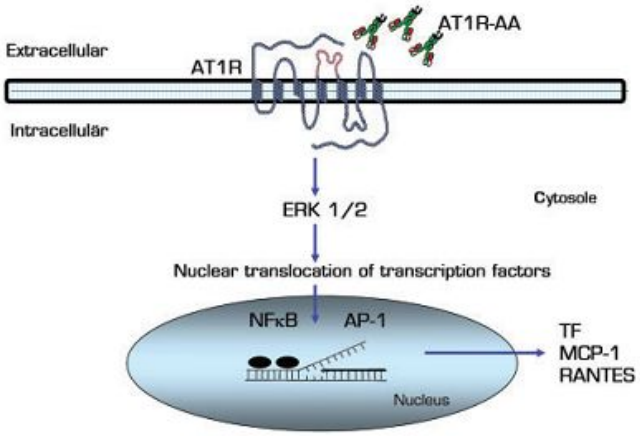


Figure 8. Model for AT_1R-Abs related signalling in vascular cells. AT_1R-Abs as a receptor agonist binds to the second extracellular loop of the AT_1R target. Receptor-antibody interaction activates downstream signalling pathways via ERK1/2 and increases expression of pro-inflammatory MCP-1 (Monocyte Chemoattractant Protein-1) and TF (tissue factor).

Considering that anti-ET_AR antibodies may also induce endothelial activation, triggering a proinflammatory, proproliferative and profibrotic response, Banasik and colleagues[88] reported that ET_AR-Abs were associated with a degradation of the transplant function during the first year after transplantation. ET_AR-Abs may thus play a crucial role between the intravascular and interstitial renal compartment in the vascular rejection[88]. In other studies, influence of the agonistic antibodies anti-AT_1R, and anti-ET_AR among patients with stable renal function was assessed five years after transplantation. These antibodies were depicted as the major cause of allograft rejection and ET_AR-Abs were strongly associated with AT_1R-Abs[82,89].

1.4.1.2 AT_1R-Abs and ET_AR-Abs in heart transplantation

In heart transplant recipients, endothelial activation is the key event in the development of allograft vasculopathy, a major factor for morbidity and mortality in the long-term follow-up[90]. Endothelial activation has been shown to be associated with microvasculopathy and with acute cellular rejection[91]. Hiemann et al.[11] reported that heart transplant patients with high levels of AT_1R-Abs and ET_AR-Abs more frequently presented cellular rejection and AMR than patients with low levels of agonistic antibodies. They also demonstrated a strong correlation between AT_1R-Abs and ET_AR-Abs levels at all the time points monitored and that elevated levels of AT_1R-Abs and ET_AR-Abs persistently correlated with microvasculopathy and graft loss after heart transplantation, implicating relevant effects on post transplantation morbidity and mortality[11].

Additionally, people with a heart failure due to the lack of suitable grafts for heart transplantation are usually treated by ventricular assist devices (VADS) which are associated with acute and chronic rejection[92]. Salisch et al.[93] showed for the first time the incidence of AT_1R-Abs and ET_AR-Abs in VADS and suggested that AT_1R-Abs and ET_AR-Abs were biomarkers for the identification of patients with high risk for rejection and microvasculopathy.

1.4.2 AT_1R-Abs and ET_AR-Abs in systemic sclerosis

A triad of vasculopathy, autoimmunity and fibrosis is a distinctive feature of SSc[12]. Activation of the immune system has a crucial role in SSc pathophysiology and auto-antibodies contribute to an inflammatory and profibrotic environment in perivascular tissue inflammation which may appear before any histological evidence of fibrosis[25,44]. Riemekasten et al.[12] showed that anti-AT_1R and anti-ET_AR antibodies were present in SSc, and that elevated antibodies levels in sera were correlated with major complications. A recent study reported that AT_1R-Abs and ET_AR-Abs were more frequent in SSc-PAH compared to other forms of pulmonary hypertension and serve as predictive and prognostic biomarkers in SSc-PAH[28]. Researchers showed that AT_1R-Abs and ET_AR-Abs were associated with fibrosis, inflammation and endothelial dysfunction either *in vitro*[12] or *in vivo*[44]. AT_1R-Abs and ET_AR-Abs can induce transforming growth factor β gene 1 (TGFβ1) expression and stimulate phosphorylation of ERK1/2 kinase in microvascular endothelial cells (HMECs)[12]; they induce proinflammatory chemokine interleukin-8 (IL-8) and vascular cell adhesion molecule-1 (VCAM-1) in HMEC-1, and similar results were obtained *in vivo*[44]. Therefore, autoantibodies directed against AT_1R and ET_AR in patients with SSc may

contribute to disease pathogenesis and could represent a link between autoimmunity, endothelial injury and fibrosis.

2 Hypothesis and Objectives

2.1 Hypothesis

Agonistic antibodies against Angiotensin II type 1 and Endothelin-1 type A receptors occur simultaneously in immune-mediated obliterative vasculopathy, like graft rejection and systemic sclerosis. These antibodies trigger a stronger and longer activation of the receptors than the endogenous ligands. Therefore, it was hypothesized that AT_1R- and ET_AR-Abs can induce the formation of heterodimers of the two receptors triggering specific intracellular signaling pathways. Moreover, considering that both receptors share a similar secondary structure, it was suggested that immune and endogenous stimuli may have different activating sites.

2.2 Objectives

In order to study the receptors, a molecular toolbox was created comprising stable cell lines expressing AT_1R and ET_AR individually or dually. Endogenous receptor agonists, ET-1, Ang II and specific pharmacologic antagonists (inverse agonists) were used to verify the functionality of the tagged receptors. These cell lines were first used to verify that the activation of the receptors is really immune-mediated.

To study the putative receptors' interaction in the models, co-immunoprecipitation studies were realized to detect AT_1R and ET_AR interaction.

In the final step, GPCR activation assay based on a yeast growth model was developed successfully. Human AT_1R or ET_AR were introduced into the yeast to enable structural-functional studies. To investigate the effects of immune stimulation on the structure of the receptors, the preliminary step was to generate second extracellular loop (ECL2) mutations of the receptors and observe their influence on the receptor functioning stimulated with patient IgG or the endogenous ligands.

3 Materials and methods

3.1 Materials

3.1.1 Chemical substances

Chemicals and Solutions	Manufacturer
3-amino-1,2,4-triazole	Sigma
Acrylamide (37.5:1) Rotiphorese® Gel 30	Carl Roth
Agarose	Serva
Albumin Fraction V, BSA	Carl Roth
Ammonium persulfate (APS)	Sigma Aldrich
Ampicillin	Alkom
Bacto Agar	BD
Bacto Trypton	BD
Bacto Yeast Extract	BD
BICIN	Carl Roth
Bis-Tris	AppliChem
Bromphenol blue	Sigma Aldrich
Blasticidin S (selective antibiotic for the bsr or BSD genes)	InvivoGen
Chloroform	Merck
Diethylpyrocarbonate (DEPC)	Sigma Aldrich
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	AppliChem
DMEM(Dulbecco's Modified Eagle's medium) 4.5g/L Glucose	PAA
DMEM 1.5 g/L Glucose	PAA
dNTPs	Promega
dNTP Mix	Applied Biosystems
Dithiothreitol (DTT)	Sigma-Aldrich
Dulbecco’s Phosphate Buffered Saline (PBS)	Biochrom
Dextrose (D(+)-Glucose)	Applichem
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth
Ethidium bromide	Sigma Aldrich
Ethanol 99.8%	Carl Roth
Ethanol 96% (MEK, denaturated)	Herbeta Arzneimittel
Fetal bovine serum (FBS)	Gibco
FDGlu(Fluorescein-D-glucopyranoside)	Invitrogen
Glycerine	Carl Roth
Glycine	Serva
G418 (selective antibiotic for the neo gene)	InvivoGen
ß-Glycerophosphate disodium salt hydrate	AppliChem
HEPES	Carl Roth
Hydrochloric acid (37%)	Carl Roth
Isol-RNA Lysis Reagent	5Prime
1 kb DNA ladder	Fermentas St. Leon-Rot
IGEPAL CA-630 (NP-40)	Sigma Aldrich
L-Glutamine	PAA
L-arginine (HCl)	Applichem
L-aspartic acid	Applichem
L-glutamic acid (monosodium)	Applichem

Chemicals and Solutions	Manufacturer
L-lysine monohydrate	Applichem
L-methionine	Applichem
L-phenylalanine	Applichem
L-serine	Applichem
L-threonine	Applichem
L-tyrosine	Applichem
L-valine	Applichem
L-histidine	Applichem
Lithium acetate	Applichem
Methanol	Carl Roth
MOPS	Applichem
M-MuLV Reverse Transcriptase	BioLabs
Nonfat dried milk powder	AppliChem
NaF (Sodium Fluoride)	Applichem
N-lauroyl-Sarkosine	Sigma
3-(N-morpholino)-propanesulfonic acid (MOPS)	AppliChem
Opti-Minimal Essential Medium (MEM)	Gibco
Penicillin/Streptomycin (P/S)	PAA
2-Propanol	Carl Roth
Protease Inhibitor Tablets, Complete Mini	Roche Applied Science
Protein Marker VI (10-245) prestained	AppliChem
Ponceau S	Carl Roth
Protein G Sepharose 4 Fast Flow	GE Healthcare
PVDF (polyvinylidene difluoride)	GE Healthcare
PEG 3350	Sigma-Aldrich
RNase Inhibitor	Applied Biosystems
Roche complete	Roche Diagnostics GmbH
SeaKem LE Agarose	Lonza
Sodium azide	Sigma Aldrich
Sodium chloride	Carl Roth
Sodium hydroxide	Sigma Aldrich
Sodium orthovanadate (Na_3VO_4)	Roth
Sodium pyrophosphate	Applichem
Sodium dodecyl sulfate (SDS) Pellets	Carl Roth
SuperSignal West Pico	Thermo Scientific
SuperSignal West Dura	Thermo Scientific
ssDNA(single-stranded DNA)	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Trypsin-EDTA (10x)	PAA
Tris	Carl Roth
Tween®20	Carl Roth
Tris-HCl	Sigma Aldrich
Triton X-100	Sigma
Trypan blue	Sigma
Trizol	Invitrogen
Yeast nitrogen base without amino acids (AA)	Sigma-Aldrich

3.1.2 Kits

Kits	Manufacturer
Anti-His Antibody Selector Kit	Qiagen
DC™ Protein Assay	Bio-Rad
In-Fusion ® HD Cloning Kit	Clontech
FastStart Universal SYBR Green Master (Rox)	Roche
NucleoBond®Xtra Midi/Maxi	Clontech
Plasmid DNA Purification Kit	Macherey-Nagel
PeqGOLD MicroSpin Gel Extraction Kit	PEQLAB
Pierce™ F(ab´)2 Preparation Kit	Thermo Scientific
Pow Sybr® Green PCR Master Mix	Applied Biosystem
Q5® Site-Directed Mutagenesis Kit	New England BioLabs
Q5® High-Fidelity DNA Polymerase	New England BioLabs
TurboFect™Transfection Reagent	Thermo Scientific
Zyppy™ Plasmid Miniprep Kit	Epigenetics

3.1.3 Plasmid, Bacteria, and Enzymes

Plasmid	Manufacturer
pSELECT-NHis-blasti	InvivoGen
p3xFLAG-CMV-10	Sigma Aldrich
p426 GPD	Glaxosmithkline

Bacteria	Manufacturer
NEB 5-alpha Compent E.coli	NEB
Stellar competent Cells	Clontech

Enzymes and provided buffer	Manufacturer
BamHI-HF	New England BioLabs
BSA	New England BioLabs
ClaI	New England BioLabs
EcoRV-HF	New England BioLabs
EcoRI-HF	New England BioLabs
HindIII	New England BioLabs
NcoI	New England BioLabs
NEBuffer 4	New England BioLabs
NEBuffer 2	New England BioLabs

3.1.4 Agonists and Antagonists

Agonists and Antagonists	Manufacturer
Angiotensin II human	Sigma Aldrich
BQ123	Sigma-Aldrich
Endothelin 1 human, porcine	Sigma Aldrich
Valsartan	Novartis

3.1.5 Buffer recipes

Buffer	Reagent	Final Conc.
3-AT	3-amino-1,2,4-triazole	1 M
Blocking buffer	Nonfat dried milk powder	5% m/v
	BSA	1% m/v
	ad 1x TBS-T	
Binding buffer, pH 7.0	Na_2HPO_4	0.02 mol/L
BICIN transfer buffer 1x	BICIN	25 mM
	Bis-Tris	25 mM
	EDTA, pH 8.0	1 mM
	Ethanol	10% v/v
BU salts 10x, pH 7.0	$Na_2HP0_4{\cdot}7H_2O$	70% m/v
	NaH_2PO_4	30% m/v
	Sterilize by autoclaving	
DNA sample buffer 6x	Glycerol	30% v/v
	Xylene cyanole	0.25% v/v
	Bromphenolblue	0.25% m/v
Elution buffer, pH 2.7	Glycin-HCl	0.1 mol/L
FDGlu assay medium, pH 7.0	YNB 10x	10%v/v
	40% glucose	5%v/v
	BU salts10x	10%v/v
	FDGlu	10 µM
	3-AT	2 mM
	ad WHAUL medium	
Gel buffer 3.5x, pH 6.5-6.8	Bis-Tris	1.25 M
Glycine stripping buffer, pH 2.0	Glycine	25 mM
	SDS	1% m/v
HEPES-NaCl buffer, pH 7.5	HEPES	10 mM
	NaCl	150 mM
Laemmli buffer 5x	Tris-HCl, pH 7.5	250 mM
	DTT	500 mM

Buffer	Reagent	Final Conc.
	Glycerol	30% v/v
	SDS	5% m/v
	Bromphenol blue	0.25% m/v
Lysis buffer (Mini-prep)	NaOH	200 mM
	SDS	1% m/v
Lysogeny broth (LB) medium	Bacto tryptone	1% m/v
	Bacto yeast extract	0.5% m/v
	NaCl	1% m/v
	Sterilize by autoclaving	
LB plate	Bacto tryptone	1% m/v
	Bacto yeast extract	0.5% m/v
	Bacto agar	1.5% m/v
	NaCl	1% m/v
	Sterilize by autoclaving	
LiAc	lithium acetate	1 M
	Filter sterilized	
LiAc-TE	LiAc	0.1 M
	TE 10x	10%v/v
LiPEG-TE	LiAc	0.1 M
	TE 10x	10%v/v
	50% PEG	80% v/v
MOPS running buffer 1x, pH 7.7	MOPS	50 mM
	Tris	50 mM
	EDTA, pH 8.0	1 mM
	SDS	0.1% m/v
Neutralization buffer (Mini-prep)	Potassium acetate	3 M
	Acetic acid	11.5% v/v
50/O/5/2-lysis buffer, pH 7.4	Tris	1 M
	EDTA	500 mM
	EGTA	500 mM
	SDS	10% m/v
50% PEG	PEG3350	50% m/v

Buffer	Reagent	Final Conc.
	Filter sterilized	
PBS (Ca^{2+}- /Mg^{2+} -free), pH 7.3	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	9 mM
	KH_2PO_4	2.3 mM
RIPA lysis buffer (normal)	Tris-HCl, pH 8.0	20 mM
	NaCl	150 mM
	EDTA	5 mM
	NP-40	1% v/v
	Sodium deoxycholate	0.5% v/v
	SDS	0.1% m/v
	Roche Complete	0.1% v/v
	β-Glycerolphosphate	10 nM
	NaF	10 nM
	Na_3VO_4	1 nM
	Sodium pyrophosphate	10 nM
RIPA lysis buffer (IP)	Tris-HCl, pH 8.0	20 mM
	NaCl	150 mM
	NP-40	1% v/v
	Sodium deoxycholate	0.5 % v/v
	SDS	0.1 % m/v
	Roche Complete	0.1% v/v
	β-Glycerolphosphate	10 nM
	NaF	10 nM
	Na_3VO_4	1 nM
	Sodium pyrophosphate	10 nM
Resuspension buffer (Mini-prep)	Glucose	50 mM
	Tris-Cl, pH 8.0	25 mM
	EDTA, pH 8.0	10 mM
	RNase A	1% v/v
Sarcosyl solution	N-lauroyl sarkosine	0.2% v/v
	ad 1x TBS buffer	
TAE buffer 1x, pH 8.4	Tris	40 mM
	Acetic acid	20 mM
	EDTA, pH 8.0	1 mM

Buffer	Reagent	Final Conc.
TBE buffer 1x, pH 8.0	Tris	89 mM
	Boric acid	89 mM
	EDTA	2 mM
TBS-T buffer 1x, pH 7.6-8.0	Tris	50 mM
	NaCl	150 mM
	Tween 20	0.1% v/v
TE buffer 10x	Tris-HCl, pH 7.5	0.1 M
	EDTA	0.01 M
TST lysis buffer	Tris-HCl, pH 8.0	100 mM
	SDS	0.2% m/v
	TritonX-100	1% v/v
	Roche Complete	0.1% v/v
	β-Glycerolphosphate	10 nM
	NaF	10 nM
	Na_3VO_4	1 nM
	Sodium pyrophosphate	10 nM
WHAUL powder	L-arginine (HCl)	1.2 g
	L-aspartic acid	6.0 g
	L-glutamic acid (monosodium)	6.0 g
	L-lysine	1.8 g
	L-methionine	1.2 g
	L-phenylalanine	3.0 g
	L-serine	22.5 g
	L-threonine	12 g
	L-tyrosine	1.8 g
	L-valine	9.0 g
YNB 10x	yeast nitrogen base without AA	6.7% m/v
	Filter sterilized	
WHAUL medium, pH 7.0	WHAUL powder	1.1 g
	ad distilled water	850 mL
	Sterilize by autoclaving	
WHAUL plate, pH 7.0	WHAUL powder	1.1 g
	Bacto agar	20 g
	YNB 10x	10%v/v
	40% glucose	5%v/v

Buffer	Reagent	Final Conc.
	ad distilled water	850 mL
	Sterilize by autoclaving	
YPD medium	Bacto yeast extract	1% m/v
	Bacto peptone	2% m/v
	dextrose	2% m/v
	Sterilize by autoclaving	
YPD plate	Bacto agar	2% m/v
	Bacto yeast extract	1% m/v
	Bacto peptone	2% m/v
	dextrose	2% m/v

3.1.6 Primers

Primers were generated with the Primer3 program with at least 18 nucleotides in length, GC content less than 60% and product size between 100-200 base pairs. All primers were provided by TIB Molbiol (Germany) or Biolegio (Netherlands). Primers to generate the constructs are listed in the Table 1. Primers for sequencing are listed in the Table 2. Primers for quantitative real-time PCR (qPCR) are listed in the Table 3.

Constructs Name	5'- to -3'
His-AT_1R	Forward:TCACCATCACGGATCCATTCTCAACTCTTCTACT
	Reverse:GCCAGCTAGCCCATGGAAATATAACTTTGCCAGA
AT_1R with ECL2 of ET_AR	Forward: CACATCAAAATTCATGGAGTTCTACCAAGATGTAAAGGACTGGTGGC TGGGCCTGACCAAAAATATACTGG
	Reverse: GCATTGAGCATACAGGTTTTATGCTGTTCACCCCTATATTCAAAGGGA CTGGCCAAGCCTGCCAG
ET_AR with ECL2 of AT_1R	Forward: TACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTCCCGGA TGTAAAGGACTGGTGGCTCTTCG
	Reverse: ATATTGGTGTTCTCAATGAAAAATACATTTCGATGGATCGCTTCAGGAA TGGCCAG

Table 1. Primers for constructs

Sequencing Primers	5'- to -3'
p426GPD (from nucleotides 4025 to 4046)	Forward :TTGACCCACGCATGTATCTATC
AT_1R (from nucleotides 414 to 433)	Forward :TCGACGCACAATGCTTGTAG
AT_1R (from nucleotides 846 to 866)	Forward :GCCATGCCTATCACCATTTG
ET_AR (from nucleotides 446 to 465)	Forward :ATCACAATGACTTTGGCGTA
ET_AR (from nucleotides 715 to 734)	Forward :TGTATGCTCAATGCCACATC

Table 2. Primers for sequencing

Gene	5'- to -3'
AT_1R	Forward: AGTCGGCACCAGGTGTATTT
	Reverse: ATCACCACCAAGCTGTTTCC
ET_AR	Forward: GATAGCCAGTCTTGCCCTTG

	Reverse: CAGAGGTTGAGGACGGTGAT
GAPDH	Forward: CATCACCATCTTCCAGGAGCG
	Reverse: TGACCTTGCCCACAGCCTTG
B_2M (Beta-2-Microglobulin)	Forward: CTGCTACGTAACACAGTTCCACCC
	Reverse: CATGATGCTTGATCACATGTCTCG

Table 3. Primers for qPCR

3.1.7 Media

Complete growth Medium

Component	Conc.
DMEM (4.5g/L glucose)	
FBS	10% v/v
Penicillin/Streptomycin (P/S)	100 U/mL
L-Glutamine	2 mM
HEPES, pH7.3	10 mM
sodium pyruvate	1 mM

Starvation Medium

Component	Conc.
DMEM (4.5g/L glucose)	
FBS	1% v/v
Penicillin/Streptomycin (P/S)	100 U/mL
L-Glutamine	2 mM
HEPES, pH7.3	400 nM
sodium pyruvate	1 mM

Freezing Medium

Component	Conc.
Complete growth Medium	50 mL
FBS	10% v/v
DMSO	10% v/v

3.2 Methods

3.2.1 Generation of constructs

3.2.1.1 cDNA fragment amplification by PCR

The target cDNA fragment was amplified using specific primers shown in Table 1. PCR relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. The Q5®High-Fidelity DNA Polymerase was used according to the manufacturer's instructions. The reaction components for a PCR are described in Table 4. The different target genes were amplified under different conditions (Table 5 and Table 6). For generation of the loop-swapped constructs, the corresponding PCR products were treated with the Q5® Site-Directed Mutagenesis Kit according to the manufacturer’s instructions.

Component	Final Conc.
5x Q5 Reaction Buffer	1x
10 mM dNTPs	200 µM
10 µM Forward Primer	0.5 µM
10 µM Reverse Primer	0.5 µM
Template DNA	up to 1000 ng
Q5 High-Fidelity DNA Polymerase	0.02 U/µL

Table 4. The reaction components

Construct	DNA template	DNA quantity	Primers conc.
His-AT_1R	AT_1R cDNA	20 ng	10 µM
AT_1R with ECL2 of ET_AR	p426 GPD AT_1R plasmid	2 ng	1 µM
ET_AR with ECL2 of AT_1R	p426 GPD ET_AR plasmid	10 ng	10 µM

Table 5. Components of PCR reactions for the different constructs

Construct	initial denaturation	second denaturation	annealing	elongation step	final elongation	amplification cycles
His-AT_1R	98°C 30 s	98°C 10s	60.7°C 30 s	72°C 4 min	72°C 2 min	35
AT_1R with ECL2 of ET_AR	98°C 30 s	98°C 10 s	57°C 30 s	72°C 4 min	72°C 2 min	35
ET_AR with ECL2 of AT_1R	98°C 30 s	98°C 10 s	72°C 30 s	72°C 4 min	72°C 2 min	35

Table 6. PCR conditions for the different constructs

3.2.1.2 Vector's restriction

The plasmid necessary for the cloning, also called vector, was digested using restriction enzymes. Briefly, 2 µg of plasmid was digested with two restriction enzymes in the appropriate incubation buffer in a 25 µL reaction. If required, 10 mg/mL BSA was added to the sample. Samples were incubated at 37°C for three hours and then run on an agarose gel. Table 7 summarises all restriction enzymes used and their reaction conditions. The incubation buffer was adapted according to the tool double digest finder:

https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder.

Double digestion		Temp.	BSA (10 mg/mL)	Buffer
BamHI-HF™	NcoI-HF™	37°C	No	NEBuffer 4
BamHI-HF™	ClaI	37°C	Yes	NEBuffer 4
HindIII	EcoRI-HF™	37°C	No	NEBuffer 2

Table 7. Restriction endonucleases

3.2.1.3 Agarose gel electrophoresis

1.5% agarose gels were prepared in 1 x TBE. Ethidium bromide was added to give a 0.5 µg/mL final concentration. When solid, gels were placed into a gel electrophoresis apparatus and filled with 1 x TBE. The DNA samples were mixed with 6x agarose gel loading buffer to 1x final. In addition to samples, a DNA marker (1 kb DNA ladder) was run as an indicator of molecular

weight. Gels were electrophoresed at 80 V for approximately one hour. Gels were examined under ultraviolet light at 302 nm using an UV-transilluminator.

3.2.1.4 Purification of the digested vector

After vector digestion, the linearized vector was purified in order to remove the unnecessary and unspecific DNA fragments using the MicroSpin DNA Gel Extraction Kit according to the manufacturer's instructions.

3.2.1.5 Cloning of the amplified DNA fragments into the vector

In-Fusion HD Cloning Kits were designed for fast, directional cloning of one or more fragments of DNA into any vector. The enzyme included in the kit could fuse DNA fragments and linearized vectors, efficiently and precisely, by recognizing a 15 bp overlap at their ends. This 15 bp overlap could be included when designing primers for amplification of the desired sequences. The Cloning Enhancer in the kit could remove excess template DNA and PCR residue, eliminating the necessity to purify the PCR insert prior to cloning. The procedure was performed according to the manufacturer's instructions. .

3.2.1.6 Bacteria transformation

Vectors employed for making recombinant plasmids contain antibiotic resistance genes to make the bacteria selection easier. Bacteria listed in Table 8 were used for the experiments. 2.5 µL of the cloning product was added to 50 µL of bacteria and the mix was incubated for 30 minutes on ice. To create a thermal shock, the tube was placed first in a 42°C hot water bath for a time corresponding to the manufacturer's protocol and then on ice. The transformed bacteria were incubated in LB medium at 37°C under shaking for one hour. Afterwards, the bacteria were plated on selection plates and incubated overnight. The following day, several colonies could be visualized if the transformation was successful.

Bacteria	Providers	Genotype
Stellar™ Competent Cells	Clontech	*F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZ Δ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ-*
NEB 5-alpha Compent E.coli	NEB	*fhuA2, Δ (argF-lacZ) U169, phoA, glnV44, Φ 80 Δ (lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*

Table 8. List of bacteria names, providers and genotypes

Glycerol stocks enable long-term storage of bacteria, while agar plates can only be used for short-term storage. For bacterial glycerol stocks, preculture of LB medium containing the appropriate selective antibiotic was inoculated with a single potential colony picked from a

freshly streaked agar plate and shaken overnight at 37°C. 200 µL of the overnight culture was added to 800 µL of 50% glycerol in a cryovial and gently mixed. Then the glycerol stock cryovial was frozen at -80°C. To recover bacteria from glycerol stock, a sterile loop was used to scrape some of the frozen bacteria off the top of the opened cryovial and was streaked onto a new LB plate. The plate was incubated overnight at 37°C for the bacteria to grow.

3.2.1.7 Mini-prep

Standard procedure developed by Birnboim and Doly[94] was used to extract plasmid DNA from bacterial cell suspensions. A preculture of LB medium containing the appropriate selective antibiotic was inoculated with a single colony picked from a freshly streaked agar plate and cultivated at 37°C for eight hours. 2 mL of the preculture was centrifuged. The pellet was resuspended in resuspension buffer and vortexed. Lysis buffer was added and mixed well. Then neutralization buffer was added to stop the reaction. After centrifugation, the supernatant was transferred into a new tube. Ice-cold 100% ethanol was added for DNA precipitation. Supernatant was discarded after centrifugation and the pellet was washed with 70% ethanol. After centrifuging to collect the DNA in the pellet, the DNA was dried on a heat-block at 56°C. Tris buffer was used to resuspended the DNA pellet. The nucleic acid concentration was measured by NanoDrop® Spectrophotometer. The plasmids were stored at -20°C.

3.2.1.8 DNA sequencing and alignment

The plasmids potentially containing the insert were sequenced by LGC Standards (UK). The resulting sequence was analyzed by using DNA Dynamo sequence analysis software (UK) and Clustal X2 (available from the Clustal Homepage or European Bioinformatics Institute FTP server).

3.2.1.9 Midi-prep

In order to produce large amounts of the constructs, a midi-prep was made. A large bacteria culture containing the appropriate selective antibiotic and 1/1000 diluted preculture was cultured overnight at 37°C. The NucleoBond® Xtra Midi/Maxi kit was used to extract the plasmid DNA from the cultures. The procedure was performed according to the manufacturer's instructions. Tris buffer was used to elute DNA. The nucleic acid concentration was measured by NanoDrop® Spectrophotometer. The plasmids were stored at -20°C.

3.2.2 Cell culture models

3.2.2.1 Human Embryonic Kidney (HEK 293) cells

HEK 293 cells were grown in flasks with complete growth medium in a humidified atmosphere with 5% CO_2 at 37°C. Passaging was done at 80% confluence. Briefly, after washing with PBS, cells were incubated with trypsin at 37°C until the cell layer was dispersed. Cells were harvested in complete growth medium to stop the proteolytic activity of trypsin. The suspension was centrifuged at 850rpm for three minutes at room temperature. After aspiration of the supernatant, the cells were resuspended and counted in a Neubauer chamber cell to obtain the average number of cells contained in 1 mL of medium. The cells were then distributed to the different cell culture dishes or micro-well plates for the experiments (Table 9) and in a cell culture flask for further passaging. On the following day, cells were checked visually for adherence and viability.

In order to be frozen, the cells were trypsinized and harvested as described previously. After aspiration of the supernatant, the cells were resuspended in a freezing medium. The cell suspension was transferred into cryovials with a density 3.0 x 10^7 cells/mL. The stock cryovials were stored in a Cryo container overnight at -80°C, and then transferred to a liquid nitrogen container for long term storage. To thaw the cells, the stock cryovial was transferred from the nitrogen freezer to a 37°C incubator. Then the cells were resuspended with complete growth medium and transferred into a T75 cell culture flask.

	Surface Area (cm^2)	Seeding Density	Growth Medium (mL)
Dishes			
60mm	21	0.8 x 10^6	5
100mm	55	2.2 x 10^6	10
150mm	152	5.0 x 10^6	20
Culture Plates			
6-well	9	0.3 x 10^6	2
12-well	4	0.1 x 10^6	1
24-well	2	0.05 x 10^6	0.5
96-well	0.3	0.015 x 10^6	0.1
Flasks			
T-25	25	0.7 x 10^6	2
T-75	75	2.1 x 10^6	10

Table 9. Distribution of cells

3.2.2.2 Stimuli employed for cell stimulation

3.2.2.2.1 Patient IgG isolation

IgG fractions from patients containing AT_1R-Abs and ET_AR-Abs can stimulate AT_1R and ET_AR activation according to the patients' pathologies. Immunoglobulins (Ig) were obtained by affinity chromatography on HiTrap Protein G columns. The columns are packed with a bacterial protein

with a high affinity for the Fc portion of the immunoglobulin class G (IgG) isotypes (IgG1, 2, 3, 4). The binding affinity of the Fc portion to protein G decreases with decreasing pH, i.e. bound antibodies are eluted at low pH.

The isolation of the IgG was carried out from serum or plasma of patients with renal transplant vasculopathy or scleroderma renal crisis. There was no restriction based on age or gender. 20 mL serum and/or plasma were centrifuged and filtered to eliminate the debris and diluted with the same volume of binding buffer. The HiTrap Protein G columns equilibrated with binding buffer were used for loading samples with a flow rate of 20mL/minute. This step was repeated two times to increase the yield of IgG. Subsequently, the column was washed with binding buffer and IgGs were eluted with elution buffer. Fractions were pooled and then dialyzed with DMEM (1.5 g/L Glucose) for 24 hours. After dialysis, the concentration was determined in the central laboratory of Charite Berlin (CVK) and the titers of the AT_1R-Abs and ET_AR-Abs were determined by ELISA (OneLambda). Patient IgG were used as immune stimulus.

3.2.2.2.2Pepsin digestion of Patient IgG

$F(ab')_2$ fragments without Fc are smaller than whole Igs but maintain their antigen binding function. Pepsin is a non-specific endopeptidase that is active only at acid pH. Digestion of IgGs by this enzyme normally produces one $F(ab')_2$ fragment and numerous small peptides of the Fc portion (Figure 9). $F(ab')_2$ was generated from patient IgG using Pierce™ $F(ab')_2$ Preparation Kit according to manufacturer's instructions.

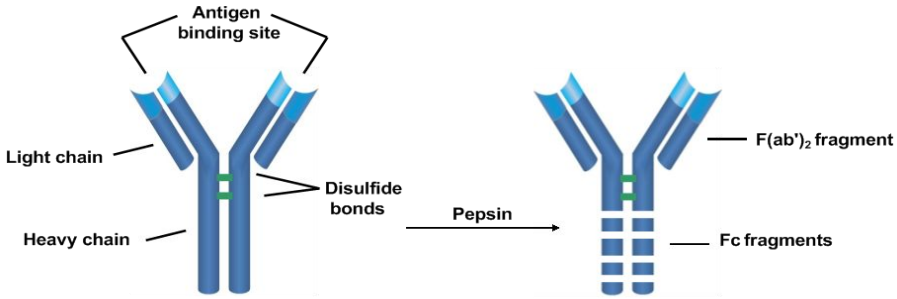


Figure 9. Digestion of IgGs to $F(ab')_2$ fragment with Pepsin. The enzyme pepsin cleaves the Fc portion of an Ig into small subfragments leaving a $F(ab')_2$ fragment with two antigen binding sites connected by disulfide bonds.

3.2.2.2.3Cell stimulation

To study the functionality of the receptors in the generated cell lines, the endogenous and immune stimuli were used to stimulate cells. 0.3 x 10^6 stable transfected cells were seeded per well and grown in a 6-well plate until 80% confluence. The cells were starved in a serum free medium overnight. The following day the cells were treated with Ang II (1000 mM), ET-1(100 mM), patient IgG (1.5 mg/mL), control IgG (1.5 mg/mL) and $F(ab')_2$ (1.5 mg/mL) for 10minutes

in the presence or absence of AT_1R blocker (Valsartan) (100 mM) or ET_AR blocker (BQ-123) (100 mM). The concentrations of the different stimuli have been established in our group previously[20].

3.2.2.3 Transient transfection

In order to analyze the expression of the generated constructs in mammalian cells, HEK293 cells were transfected transiently. 0.3 x 10^6 HEK293 cells were seeded per well in a 6-well plate and grown until 80% confluence. Then cells were transfected with the different DNA constructs independently using TurboFect™Transfection reagent following the manufacturer's instructions. The DNA was diluted in a serum-free DMEM (OptiMEM) and incubated 20 minutes. The amounts of plasmid DNA and TurboFect™Transfection reagent used are summarized in the following Table 10.

Cell culture plate	Growth area (cm^2/well)	Volume of OptiMEM (µL)	DNA (µg)	TurboFect (µL)
60 mm	21	300	2.5	5
100 mm	55	500	6	12
6-well	9	150	1	2
24-well	2	20	0.5	1
96-well	0.3	5	0.08	0.16

Table 10. Amounts of plasmid DNA and reagent used for transient transfection

3.2.3 Generation of stable cell lines

3.2.3.1 Establishment of the optimal antibiotic concentration for clone selection

In order to establish the optimal antibiotic concentration to select the transfected cells, 1.0x10^5 cells were seeded per well into 12 well-plates, and when cells were at 80% confluence, different concentrations of antibiotics corresponding to the resistance gene of the construct of interest were added into the wells. The cells were examined for visual toxicity daily and medium was changed daily. This operation was repeated until all the cells died or after seven days if all the cells did not die. A table was generated to assess the rate of cell death and the antibiotics concentration needed to achieve complete cell death within two to three days was chosen. Table 11 and Table 12 show the concentration tested and chosen for each antibiotic (Blasticidin S and G418).

Blasticidin S (µg/mL)	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
0	-	-	-	+	++	++	++	+++
5	-	-	+	++	++	+++	+++	*
10	-	+	++	+++	+++	+++	*	
12	+	++	++	+++	+++	*		
20	++	+++	*					

Note: "+" less than 50% of the cells died. "++" between 50 and 90%. "+++" more than 90%. "*" nearly 100% cells died.

Table 11. Working conditions of Blasticidin S for selection in HEK293 cells

G418(µg/mL)	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
0	-	-	-	-	-	-	+	++
200	-	-	-	+	+	++	++	+++
500	-	-	+	+	++	+++	*	
750	-	-	+	++	+++	+++	*	
1000	-	+	+	++	+++	*		
1250	+	+	++	+++	*			
1500	+	+	++	+++	*			
2000	+	+	++	*				
Note: "+" less than 50% of the cells died. "++" between 50 and 90%. "+++" more than 90%. "*" nearly 100% cells died.								

Table 12. Working conditions of G418 for selection in HEK293 cells

3.2.3.2 Selection of single cell clones

To generate single cell clones, 2.2 x 10^6 HEK293 cells were seeded and grown in a 100-mm dish until 80% confluence. Cells were then transfected with the different DNA constructs using the TurboFect™Transfection reagent according to the manufacturer's protocol. After 48 hours of incubation, cells were trypsinized and distributed to 10 dishes (100 mm) and grown in medium containing the appropriate antibiotics at the optimal concentration for two weeks. The cells were visually examined for signs of toxicity daily and medium with antibiotics was changed daily. Cells that had not integrated the transfected plasmid died while the cells that underwent plasmid integration survived until post-transfection. The surviving cells isolated and expanded in the 100-mm dish. Clones which were visually big enough and separated from the others were trypsinized using glass cylinders and seeded in a 96-well plate with selection medium. The cells were observed daily and medium was changed daily. When these wells reached confluence, they were transferred into a 12-well plate. This operation was repeated until all the different clones were grown enough to be cultured in a T75 cell culture flask and frozen for further experiments.

3.2.3.3 Checking of the transfected plasmid expression

3.2.3.3.1 Quantitative real-time PCR

Quantitative real-time PCR was employed to measure transcription levels of ET_AR and AT_1R. 0.3 x 10^6 stable transfected cells were seeded per well in a 6-well plate until 80% confluence. A brief PBS washing was followed by adding Isol-RNA lysis reagent. Then cells were scrapped and transferred into autoclaved 1.5 mL tubes. Total RNA from the cells was isolated by standard phenol-chloroform extraction. Briefly, 0.2 mL room temperature Chloroform was added to each tube and mixed 30 seconds using a vortex mixer. Samples were then centrifuged for 15 minutes

with 12000x g at 4°C. The centrifugation resulted in a phase separation into an upper, colorless, aqueous phase containing RNA and a lower, pink, organic phase containing proteins and lipids. After collection of the upper phase, RNA was recovered from the aqueous phase by precipitation with isopropanol. After vortexing and centrifugation (12000x g, 10 minutes, 4°C), a white RNA pellet was formed at the bottom of the tube. Supernatant was carefully decanted and the RNA pellet was washed with 75% ethanol and subsequently centrifuged five minutes with 7500x g at 4°C. The RNA pellet was briefly dried and resolved in 30 µL of DEPC-treated water. Then the amount of isolated total RNA was measured with a NanoDrop® Spectrophotometer and stored at -80°C for further processing.

The integrity and overall quality of RNA was verified by agarose gel electrophoresis. The RNA was separated into two visible 18S and 21S rRNA bands and a 5.0/5.8S rRNA band could sometimes be seen. 1000 ng RNA were further reverse transcribed into complementary DNA (cDNA) (Table 13).

RT-PCR reaction mixture		**RT-PCR program**	
Component	**Final Conc.**	**Temp (°C)**	**Time(min)**
10x NEB Buffer mit MgCl2	1x	25	10
10 mM dNTP-Mix	1 mM	42	45
40 U/µL Rnase Inhibitor	0.8 U/µL	95	5
200 U/µL MuLV Rev Transc NEB	4 U/µL	4	pause
50 µmol/L Random Hexamers/Oligo $d(T)_{16}$	1 µmol/L		
RNA	1000 ng		
Adjust reaction volume with DEPC-treated water			

Table 13. RT-PCR conditions and program

After reverse transcription, the relative expression of the studied genes was determined by qPCR. qPCR analysis was carried out using Power SYBR®Green PCR Master Mix according to manufacturer's instructions. Sequences for all target gene primers are shown in Table 3. Synthesized cDNA was mixed together with the other components in a 96-well plate in a total volume of 14 µL. Samples were applied in triple. The protocol for qPCR with thermocycling conditions and program is shown in Table 14. Samples were analyzed with MxPro-Mx3005P QPCR Systems, and expression levels were normalized to the housekeeping gene.

qPCR reaction mixture		**qPCR program**		
Component	**Final Conc.**	**Temp (°C)**	**Time(sec)**	
2xSYBR® Green PCR Master Mix	0.6x	50	120	
10 µM Forward primer	0.18 µM	95	600	
10 µM Reverse primer	0.18 µM	95	15	40 cycles
10 ng/µL cDNA	1.4 ng/µL	60	60	
Adjust reaction volume with distilled water				

Table 14. qPCR conditions and program

3.2.3.3.2 Total proteins extraction

In order to analyze the protein expression from cells with transfected plasmids, total proteins were extracted. Cells were washed with ice-cold PBS. The RIPA lysis buffer (normal) was added to the plates. The cells were detached with a cell scraper and collected in tubes on ice, where they were incubated for 10 minutes to completely lyse the cells. The supernatant was collected immediately after centrifugation (14,000 rpm, 30 minutes, 4°C), and prepared for protein measurement. Quantification was done using the Bio-Rad Protein Assay kit following the protocol of the manufacturer. After protein measurement, the supernatant was mixed with Laemmli buffer 1x final and distilled water to achieve the same protein concentration and the same volume in all samples. Then the samples were heated at 95°C for five minutes and stored at -20°C.

3.2.3.3.3 Generation of membrane extracts

In order to verify that the proteins resulting from the transfection were highly expressed in the cell membrane, membrane extracts were generated. Cells were washed with ice-cold PBS. 50/O/5/2-Buffer without SDS was added to 10 mm dishes. The cells were detached with a cell scraper and collected in tubes on ice. After that, the tubes were frozen in liquid nitrogen and thawed in a 37°C heating block. This step was repeated two times. After centrifugation, the supernatant was collected as control. The pellet was resuspended in 50/O/5/2-Buffer with 1% SDS for denaturing. The supernatant containing the membrane proteins was collected immediately after other same centrifugation step. After protein measurement, the steps for sample preparation were the same as those for protein extraction.

3.2.3.3.4 Immunoprecipitation

Immunoprecipitation (IP) was done to verify that the expressed protein is a fusion of the tag and the receptor. 50 µL Protein G agarose beads was washed with cold TBS buffer and incubated with 2 µg antibodies overnight, then washed with cold TBS buffer and RIPA buffer. Cells were lysed with RIPA lysis buffer (for IP) following the total protein extraction protocol. Subsequently, 1 mg proteins from cell lysate were incubated with bead-binding antibody and gentle rotated overnight at 4°C. In parallel, a small amount of lysate was prepared with Laemmli buffer as a control for western blot. The next day, the beads were microcentrifuged for 30 seconds at 4°C and the pellet was washed three times with RIPA lysis buffer. Then the pellet was resuspended with Laemmli buffer and heated at 95°C for five minutes and loaded on a MOPS-PAGE gel. The samples were analyzed as described in the western blot part.

3.2.3.3.5Gel electrophoresis and western blot

The prepared samples were applied on a 10% Bis-Tris polyacrylamide gel (Table 15) with a protein marker (Protein Marker VI) as a ladder and run with MOPS buffer under reducing conditions in a mini PROTEAN® Tetra cell at 100 V for about 120 minutes.

10% Separation gel		**5% Stacking gel**	
Component	**Final Conc.**	**Component**	**Final Conc.**
30% Acrylamide Mix	33.8% v/v	30% Acrylamide Mix	13.5% v/v
Gel buffer 3.5 x (1.25 M)	28.8% v/v	Gel buffer 3.5 x (1.25 M)	30% v/v
10% APS	1.3% v/v	10% APS	1.5% v/v
TEMED	0.13% v/v	TEMED	0.25% v/v
Adjust volume with distilled water		Adjust volume with distilled water	

Table 15. Formulation for MOPS-PAGE gel

Subsequently the proteins were transferred to a PVDF membrane in a Trans-Blot® cell with BICIN transfer buffer at 1.0 A for two hours. The membranes were blocked with 1% BSA, 5% nonfat dried milk powder in TBS-T at room temperature for 1 hour. The membranes were then incubated overnight at 4°C with the respective primary antibody (Table 16). Next, the membranes were washed three times with TBS-T and subsequently incubated with a secondary antibody for one hour at room temperature. After three additional washing steps, the horseradish peroxidase was visualized with an enhanced chemiluminescence substrate according to the manufacturer's instructions. The development procedure was supported by the Digital images system of a GeneSys machine. For further probing of previously used PVDF membranes, chemiluminescent reaction was stopped by rinsing three times with TBS-T. Stripping antibodies off the membranes was done in Stripping buffer for 30 minutes at 52^{o}C. After further washing cycles and a blocking step, the membranes were reused for subsequent antibody probes.

Antibody	**Manufacturer**	**Dilution**
Primary antibody		
Anti-Flag, mouse, monoclonal	Sigma Aldrich	1:2000
Anti-Flag, rabbit, polyclonal	Sigma Aldrich	1:400
Mouse IgG1, mouse, monoclonal	Sigma Aldrich	1:500
Anti-GAPDH , mouse, monoclonal	Hytest	1:50000
Anti-ET-1 receptor, mouse, monoclonal	BD Biosciences	1:1500
mAnti-pp44/42 MAPK (ERK1/2), rabbit, polyclonal	Cell Signaling	1:1000
Anti-p44/42 MAPK (ERK1/2), rabbit, polyclonal	Cell Signaling	1:1000
Anti-AT_1 receptor, rabbit, polyclonal	Santa Cruz	1:400
Anti-RGS·His, mouse, monoclonal	Qiagen	1:2000
Anti- α-Tubulin, mouse, monoclonal	Sigma Aldrich	1:7500
Secondary antibody		
Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG	Dianova	1:10 000
Peroxidase-conjugated AffiniPure Donkey Anti-Rabbit IgG	Dianova	1:10 000

Table 16. Antibodies for western blot

3.2.3.4 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) analysis was used to study the interaction between the two receptors in the stable cell lines. Co-IP is based on the potential of IP reactions to capture and purify the primary target (i.e., the antigen) as well as other macromolecules that are bound to the target by native interactions in the sample solution. The experiments are based on the IP protocol. After the beads were incubated with the appropriate antibody and cell lysate, the beads were incubated with a Sarcosyl solution at 4°C for four hours, slowly rotated and then centrifuged for 60 seconds at 550 rpm at 2°C. The co-precipitated proteins were detached in the supernatant and the precipitated proteins should still be on the beads. Then the pellet was resuspended with Laemmli buffer and heated at 95°C for five minutes and loaded on a MOPS-PAGE gel. The samples were analyzed as described in the western blot part.

3.3 GPCR activation assay

The functional coupling of heterologous GPCRs to the pheromone-response pathway of the budding yeast Saccharomyces cerevisiae is well established as an experimental system for ligand identification and for measuring receptor activation. The method of yeast culture, maintenance, transformation and GPCR assay was described in detail by Dowell and Brown in Methods Mol Biol[95]. Stock solutions of yeasts (Table 17) were streaked out on a YPD plate. The plate was incubated at 30°C for approximately three days. Then a single colony was picked from the plate and put into a culture tube with 4 mL YPD medium. The culture was incubated with shaking at 200 rpm, 30°C overnight. On the next day 1.5 mL of the preculture was added to 100 mL YPD medium and incubated for three hours. After centrifuging (600x g, 2 minutes) and discarding the supernatant, the pellet was resuspend in 1 mL distilled water. After again centrifuging and discarding the supernatant, the yeast was resuspended in LiAc TE. 50 µL of the yeast was added to 5 µL of ssDNA (10mg/mL) with 2 µg of plasmid DNA and then mixed with 300µL of LiAc PEG TE. After an incubation of 10 minutes followed by a 20-minute heat shock at 42°C, 30µL of the transformation solution was plated out on WHAUL-His plates. The plates were incubated at 30°C for three days. Single colonies were picked and streaked out on a new WHAUL-His plate. The plate was incubated overnight. Yeast from the streaked out plate was added to WHAUL-His medium (WHAUL medium +10% His) in a 96 well plate for pre-culturing. The plate was incubated at 100 rpm and 30°C overnight. The experiment was performed with four different clones of each transformation, with four different concentrations of ligands or patient IgG in triplets. FDGlu medium with the different stimulus concentrations was prepared and pipetted into the 96well plates. 1 µL of the respective preculture yeast clone was added. The

plates were put into aluminum foil and incubated at 30°C for 20 hours. The plates were measured for fluorescence with an excitation of 485 nm and emission of 535 nm.

Strain	Genotype
MMY12	MMY11 TRP1::GPA1
MMY14	MMY11 TRP1::Gpa1/Gαq(5)

Table17. Genotypes of yeast strains used for GPCR research

3.4 Statistical analysis

Data are provided as means ± standard error of the mean (SEM); n represents the number of independent experiments. All data were compared using a one-way ANOVA with a Bonferroni's multiple comparison post-hoc analyses where applicable, and only results with a P value < 0.05 were considered statistically significant. All graphic and statistical analyses were performed with Graphpad Prism™ version 5.04 (USA).

4 Results

4.1 Generation of stable cell lines expressing Angiotensin II type 1 and Endothelin-1 type A receptors individually or dually

4.1.1 AT_1R over-expressing stable cell line

4.1.1.1 Generation of the AT_1R pSELECT-NHis-blasti plasmid

The pSELECT-NHis-blasti plasmid is used for expression of a gene of interest in mammalian cell lines. It contains two expression cassettes: the Blasticidin S-resistance gene (bsr) gene as a dominant selectable marker allowing selection in both mammalian cells and E. coli, and a poly-histidine (His) sequence for adding the His tag at the N terminus of the protein of interest to facilitate their detection and/or purification. (Figure 10).

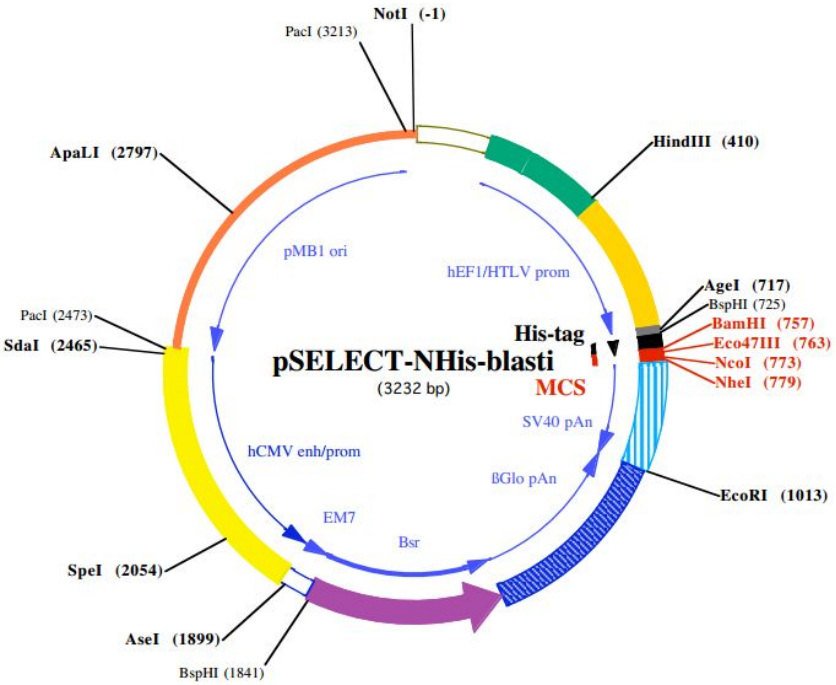


Figure 10. Map of pSELECT-NHis-blasti plasmid (3232 base pairs).

To generate the AT_1R-pSelect-Nhis-blasti plasmid, the 1080 base pair (bp)-long human AT_1R cDNA (kindly given by Pierre Lavigne, Sherbrooke University, Canada) was amplified with primers introducing two unique restriction sites BamHI and NcoI bordering the codon directly following the ATG and the stop codon (Figure 11). The resulting insert was subcloned into the pSelect NHis Blast plasmid linearized by BamHI and NcoI (Figure 12).

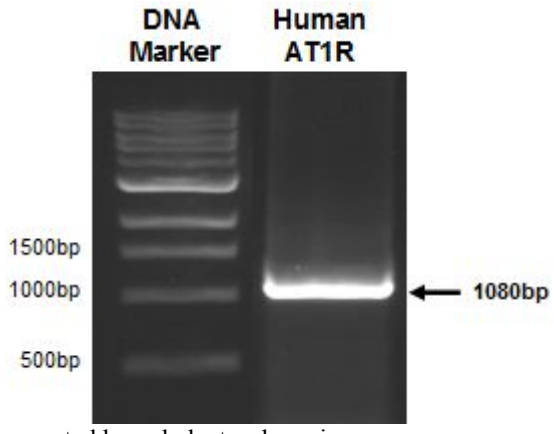


Figure 11. The amplified insert separated by gel electrophoresis.

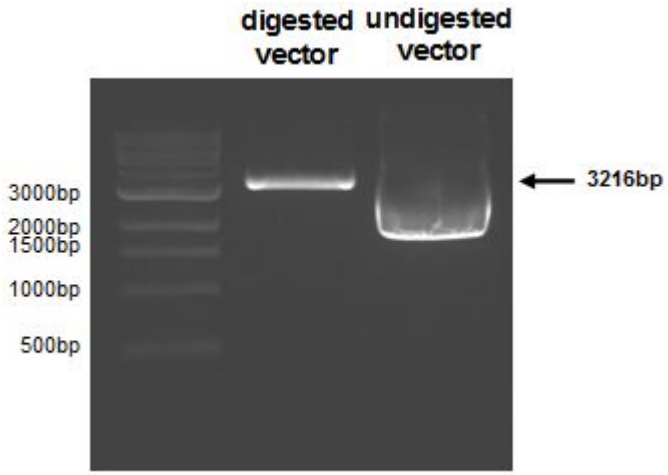


Figure 12. The digested pSelect NHis Blast plasmid separated by gel electrophoresis. The molecular weight of the linearized vector is 3216 bp without the BamHI-NcoI fragment (16 bp). The undigested vector, which as super helix structure runs faster than the digested vector, was used as a control.

Following transformation, thirty bacterial clones containing putatively the plasmid construct were analyzed with a double digestion BamHI and NcoI (Figure 13). To confirm that the target AT_1R fragment was correctly cloned into the vector, EcoRI and Hind III were chosen to double digest the plasmid. These two enzymes cut it into two fragments of 2629 and 1667 bp (Figure 14). Five clones contained the recombinant AT_1R plasmid.

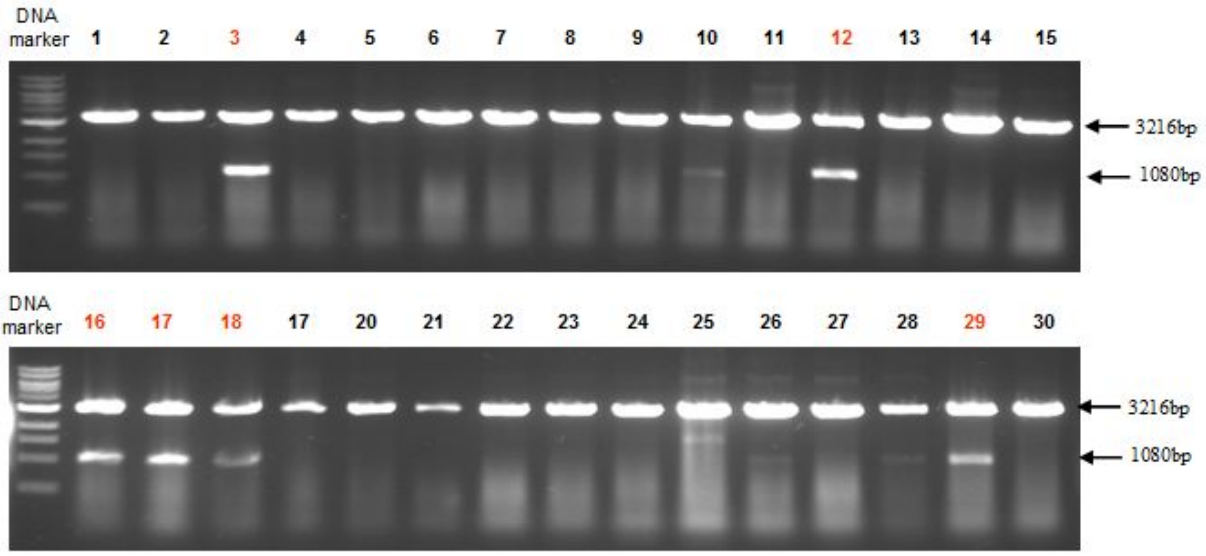


Figure 13. The double-digested thirty clones separated by gel electrophoresis. The clones containing both inserts and vector fragments are written in red.

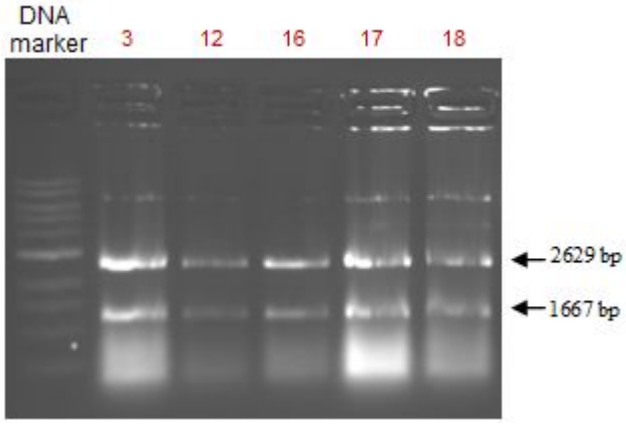


Figure 14. Tentatively identified clones were confirmed using EcoRI and HindIII restriction enzymes. The clones with red color were positive.

LGC Standards Company (UK) provided DNA sequencing of the potential clones to verify recombinant clones after cloning. Sequence information was analyzed by using DNA Dynamo sequence analysis software (UK). The plasmid successfully confirmed was used for transfection.

4.1.1.2 Generation of the His-AT_1R^+ cell line

Different concentrations of His-AT_1R plasmid were transiently transfected into the HEK293 cells. 2.5 µg His-AT_1R plasmid transfected into cells resulted in the highest His-tagged AT_1R protein expression (Figure 15). The Blasticidin S dosis test in HEK293 cells showed that the 12 µg/mL dosis was optimal to achieve a quick yet not too strong selection of the transfected cells. This dosis was used to select the cells two days after transfection. After a two-week selection, fifty two clones were picked up and further cultured.

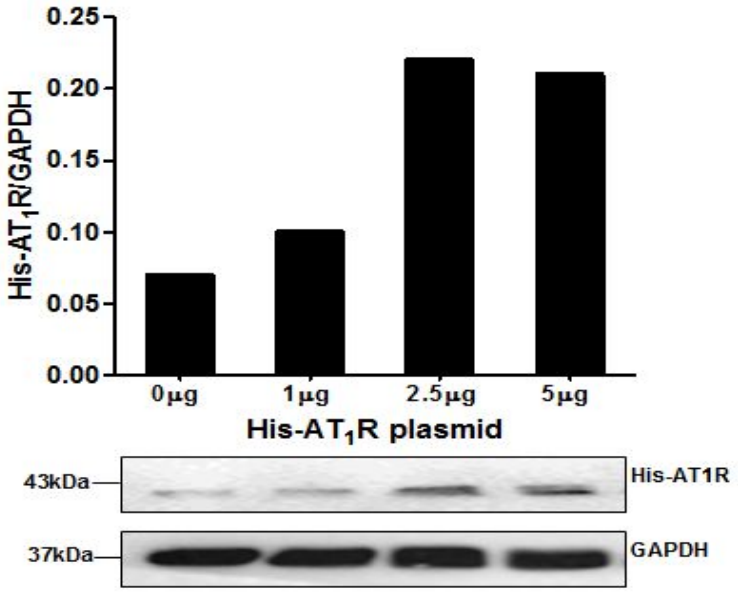


Figure 15. His-tagged AT_1R expression in transient transfected cells. The total cell extracts were analyzed by western blot with an antibody specific for AT_1R. GAPDH was used as loading control.

4.1.1.3 Characterization of the His-AT_1R^+ cell line

The 52 clones expressing possibly the His-AT_1R fusion protein were subjected to western blot analysis with an antibody directed against the His tag. As expected, expression levels were variable among the different clones, and four clones (7, 18, 51, 32) were selected for further experiments (Figure 16).

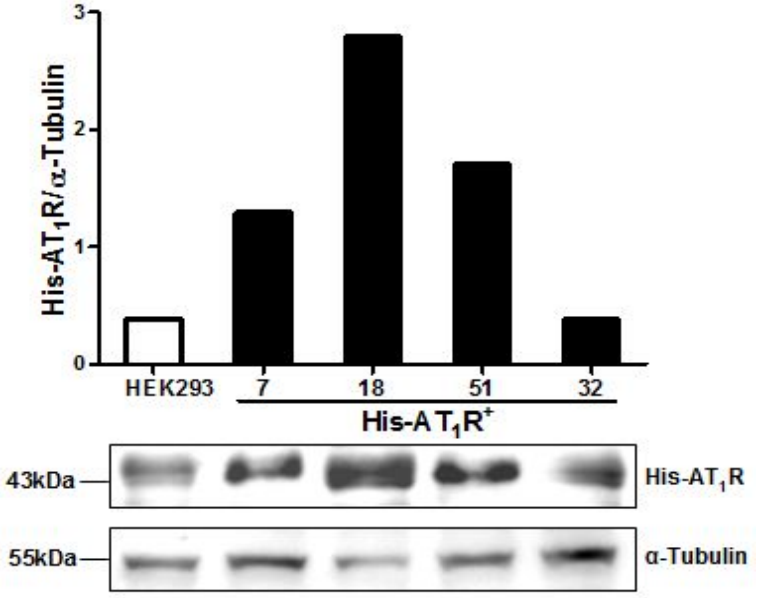


Figure 16. His-tagged AT_1R expression in His-AT_1R^+ cell clones. Total cell extracts were analyzed by western blot with an anti-His antibody. α-Tubulin was used as loading control.

AT_1R, as a transmembrane receptor, is highly expressed in the plasma membrane. In order to verify the presence of AT_1R in the cell membrane, membrane protein extracts were isolated from the four clones and western blot was performed (Figure 17). The His-AT_1R^+ cell clones

expressed higher His-tagged AT_1R protein in the membrane extracts than in the cytosolic fraction and in the non-transfected cells. These results proved that the cell clones can over-express the His tagged AT_1R protein stably and that the receptor was located at the plasma membrane. The clone 18 expressing the highest His-AT_1R levels was used for analyzing the functionality of the tagged receptor.

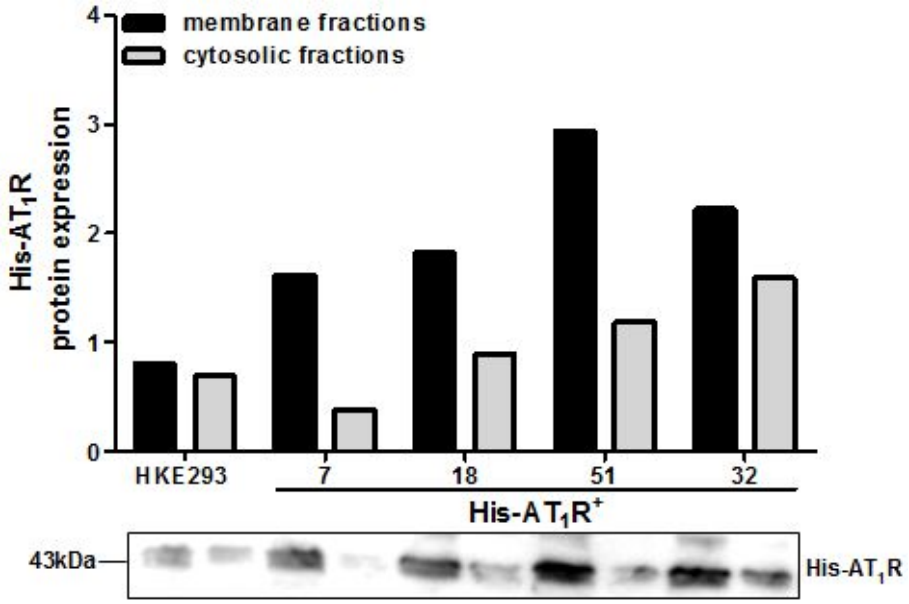


Figure 17. Western blot analysis of His-AT_1R expression in the membrane and cytosolic fractions of different His-AT_1R^+ cell lines compared to HEK293. HEK293 were used as negative controls.

4.1.1.4 Activation of the tagged AT_1R in response to endogenous and immune stimuli

The p44/42 MAPK (ERK1/2) signaling pathway can be activated in response to a wide range of extracellular stimuli, and in particular in response to the activation of AT_1R. The extracts from stimulated cells were blotted with anti-phospho-ERK antibody to monitor ERK activity. Ang II , the endogenous ligand of the Angiotensin II type 1 receptor, increased ERK1/2 phosphorylation in the His-AT_1R^+ cell line compared with non-transfected cells and the magnitude of ERK1/2-induced phosphorylation was much higher in the stable transfected cell line ($P<0.05$). To verify that the observed Ang II effect was specific to the receptor in the His-AT_1R^+ cell line, cells were pre-exposed to the selective AT_1R antagonist Valsartan (Figure 18). The pre-incubation resulted in a loss of ERK1/2 activation ($P<0.05$). Thus, the tagged AT_1R kept its endogenous function in His-AT_1R^+ cell line.

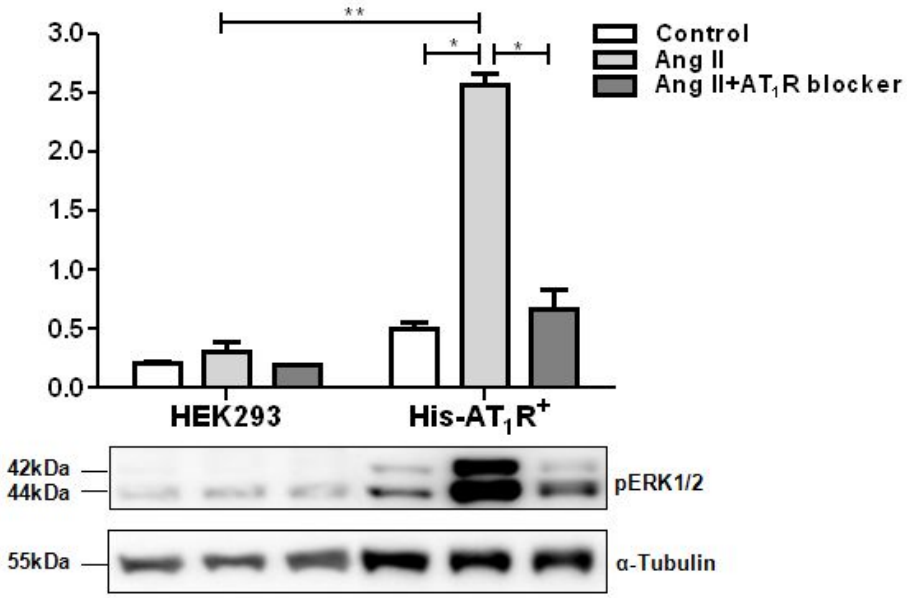


Figure 18. Activation of the tagged AT_1R in response to endogenous stimulation. Cultured cells were starved for 24h in serum-free medium. Then cells were preincubated for 1h with Ang II blocker (100 mM), and subsequently treated with Ang II (100 mM) for 10 min. Cell lysates were immunoblotted with an anti-pERK specific antibody to monitor ERK activation. α-Tubulin was used as loading control. Non-stimulated cells were used as a negative control. All experiments were repeated three times. *$P<0.05$, **$P<0.01$.

To investigate the response of AT_1R to an immune stimulation with AT_1R-Abs and ET_AR-Abs in the stable cell line, phosphorylation of ERK1/2 was examined in the His-AT_1R^+ cell line incubated with patient IgG (P-IgG). Stimulation with P-IgG induced a higher ERK1/2 phosphorylation in the His-AT_1R^+ cell line compared with non-transfected cells and non-stimulated cells (Figure 19). Thus, anti-AT_1R-Abs and anti-ET_AR-Abs can exert their biological effects on the tagged AT_1R in the His-AT_1R^+ cell line.

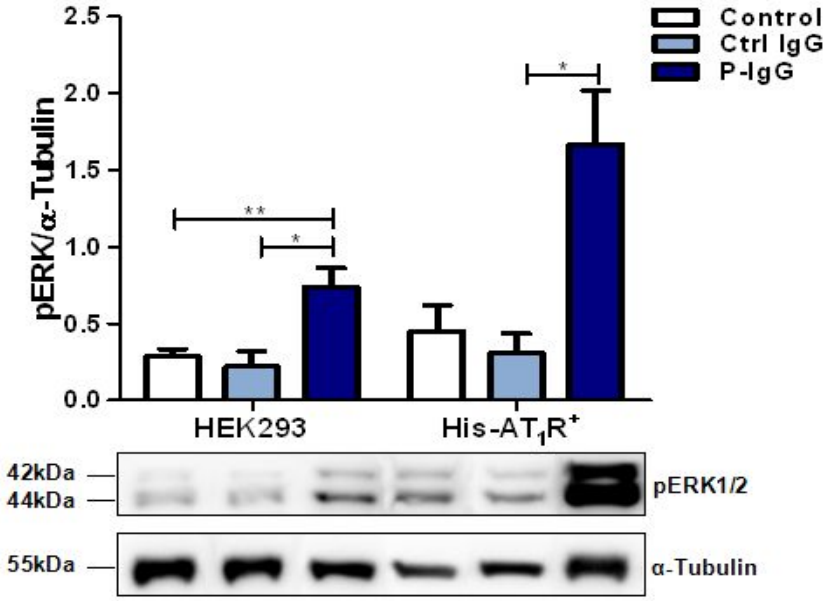


Figure 19. Activation of the tagged AT_1R in response to immune stimulation. Cultured cells were starved for 24 h in serum-free medium, and then cells were stimulated with P-IgG (1.5 mg/mL) or control-IgG for 1h. Cell lysates were analyzed by western blot with an antibody against pERK1/2. α-Tubulin was used as loading control. Non-stimulated

cells were used as control. HEK293 were used as negative controls. The experiments were repeated three times. *$P < 0.05$, **$P < 0.01$.

4.1.2 ET_AR over-expressing stable cell line

4.1.2.1 Generation of the Flag-ET_AR^+ cell line

The Flag-tagged ET_AR plasmid containing the Neomycin resistance gene (neo) gene for selection was obtained in our laboratory and was confirmed by electrophoresis and sequencing. A transient transfection of HEK293 cells was performed to determine the best quantity of plasmid for generating stable cell clones. Transfection of 2.5 μg Flag-ET_AR plasmid resulted in the highest Flag-tagged ET_AR protein expression (Figure 20). The same protocol as for Blasticidin S was used to establish the optimal G418 dosis for stable cell clones selection. The cells were selected two days after Flag-ET_AR transfection with 1000 μg/mL of G418. After a two-week selection, twenty clones were picked and further cultured.

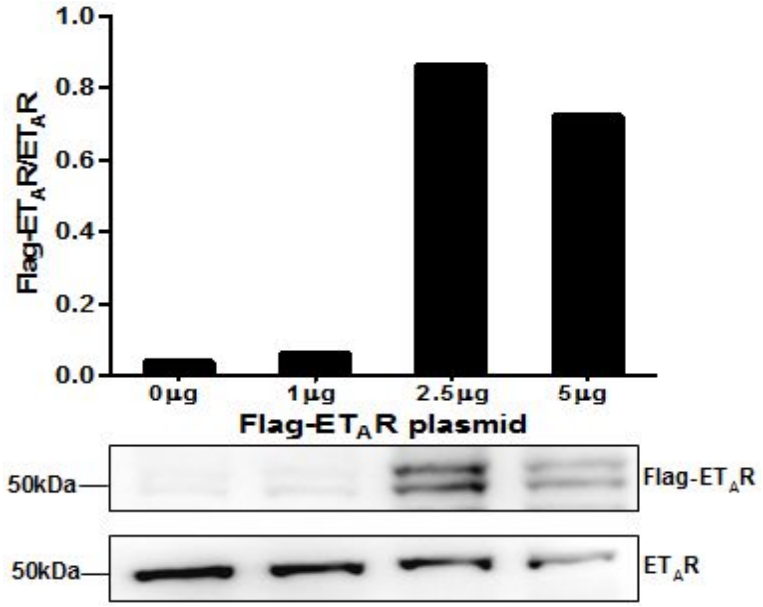


Figure 20. Flag-tagged ET_AR expression in transient transfected cells. The total cell extracts were isolated and analyzed by western blot with anti-ET_AR and anti-Flag antibodies.

4.1.2.2 Characterization of the Flag-ET_AR^+ cell line

Total cellular extracts from the twenty clones were analyzed by western blot with antibodies directed against the Flag tag. Flag-ET_AR expression levels were variable among the different clones and three clones (4, 12, 14) expressed higher levels of Flag-ET_AR in total cellular protein extracts (Figure 21). To verify the ET_AR expression in the cell membrane, western blot was performed to analyze the plasma membrane protein fractions. Figure 22 shows that the Flag-ET_AR^+ cell lines expressed higher Flag-tagged ET_AR protein in the membrane protein extracts than in the cytosolic fraction and also in comparison to the non-transfected cells. Western blot

showed that clone 14 expressed the highest Flag-ET_AR protein levels and real time PCR experiments verified that the ET_AR mRNA level of Flag-ET_AR^+ cell line in clone 14 was higher (approximately 200-fold increase) than in non-transfected cells (Figure 23). Therefore, clone 14 was used for further experiments.

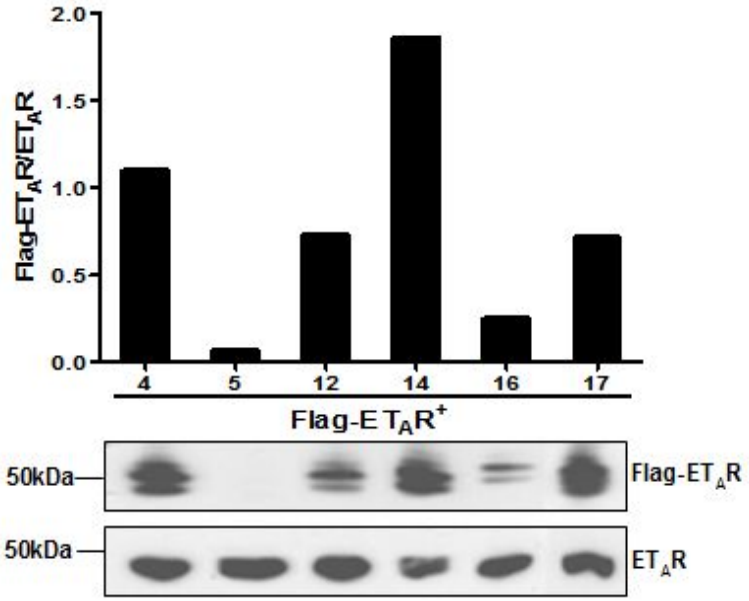


Figure 21. Flag-tagged ET_AR expression in Flag-ET_AR^+ cell clones. Cells were grown in the presence of G418. Total cell extracts were analyzed by western blot with anti-Flag and anti-ET_AR antibodies.

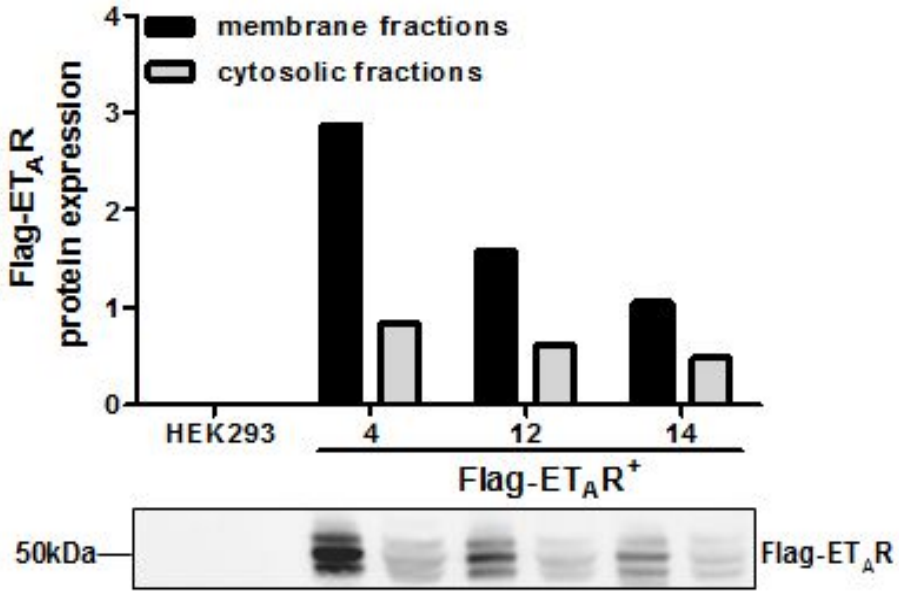


Figure 22. Western blot analysis of Flag-ET_AR expression in the membrane and cytosolic protein fractions of different Flag-ET_AR^+ cell clones. HEK293 were used as negative controls.

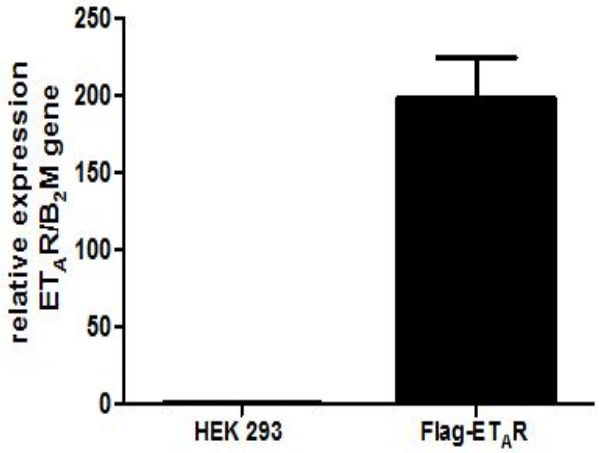


Figure 23. Real-time quantitative PCR detection for ET_AR mRNA expression in the Flag-ET_AR^+ cell line (clone 14). HEK293 were used as negative controls. Data are representative of three independent experiments.

4.1.2.3 Activation of the tagged ET_AR in response to endogenous and immune stimuli

To analyze the tagged ET_AR activation in Flag-ET_AR^+ cell line, the natural ligand of the receptor, ET-1, was used to stimulate cells, and ERK activity was monitored. Figure 24 shows that ET-1 increased ERK1/2 phosphorylation in the Flag-ET_AR^+ cell line compared with non-transfected cells. Besides, the contribution of the tagged ET_AR activation to ET-1 effects could be blocked when Flag-ET_AR^+ cells were pre-exposed to the selective ET_AR antagonist BQ123. Hence, the fusion protein was functional in the Flag-ET_AR^+ cells.

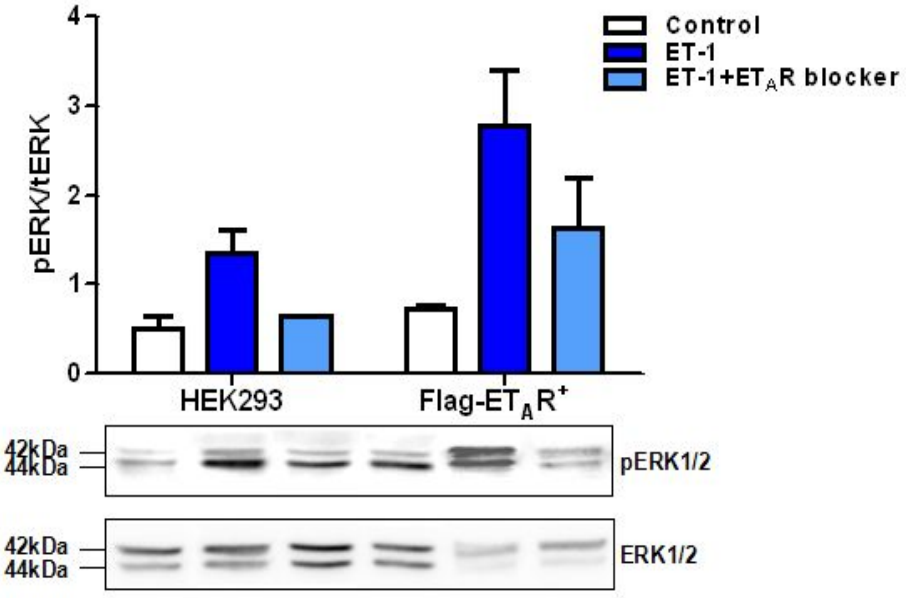


Figure 24. Activation of the tagged ET_AR in response to endogenous stimulation. Cultured cells were starved for 24 h in serum-free medium. Then cells were preincubated for 1h with ET_AR blocker (100 mM) and treated with ET-1 (100 mM) for 10 min. Cell lysates were immunoblotted with an anti-pERK specific antibody to monitor ERK activation, or with an anti-ERK antibody to confirm equivalent levels of ERK expression. HEK293 were used as negative controls. Experiments were repeated three times.

To analyze the activation of ET_AR in response to patient IgG in the Flag-ET_AR^+ cell line, phosphorylation of ERK1/2 was examined. Figure 25 shows that ERK1/2 was more phosphorylated in the Flag-ET_AR^+ cell line compared to non-transfected cells ($P<0.05$) and non-stimulated cells ($P<0.05$). Therefore, the tagged ET_AR can be induced by an immune stimulus and initiate canonical signaling mediated by ERK1/2 in the Flag-ET_AR^+ cells.

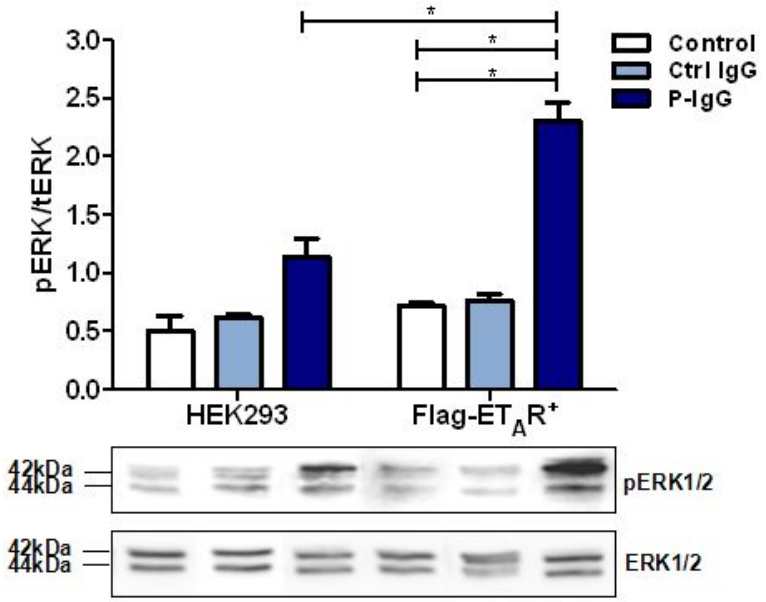


Figure 25. Activation of the tagged ET_AR in response to an immune stimulus. Cultured cells were starved for 24 h in serum-free medium, and then cells were stimulated with P-IgG (1.5 mg/mL) or control-IgG for 1h. Cell lysates were analyzed by western blot with antibodies against pERK1/2 and ERK1/2. Non-stimulated cells were used as control. HEK293 were used as negative controls. Experiments were repeated three times. *$P<0.05$.

4.1.3 ET_AR and AT_1R over-expressing stable cell lines

4.1.3.1 Generation and characterization of the Flag-ET_AR^+-His-AT_1R^+ cell lines

The dual receptor cell line was based on the Flag-ET_AR^+ cell line which was transfected with 2.5μg His-AT_1R plasmid. Blasticidin S and G418 were given continuously for maintaining selective pressure for His-AT_1R and Flag-ET_AR gene expression.

The cells were selected the same way as the individual His-AT_1R^+ and Flag-ET_AR^+ cell lines. His-AT_1R and Flag-ET_AR fusion protein expression in the dual cell line was analyzed by western blot with antibodies directed against the His tag and Flag tags. Figure 26 shows that the expression levels were different among the different clones, and three clones (6, 22 and 32) expressing the highest levels of tagged ET_AR and AT_1R were selected for further experiments.

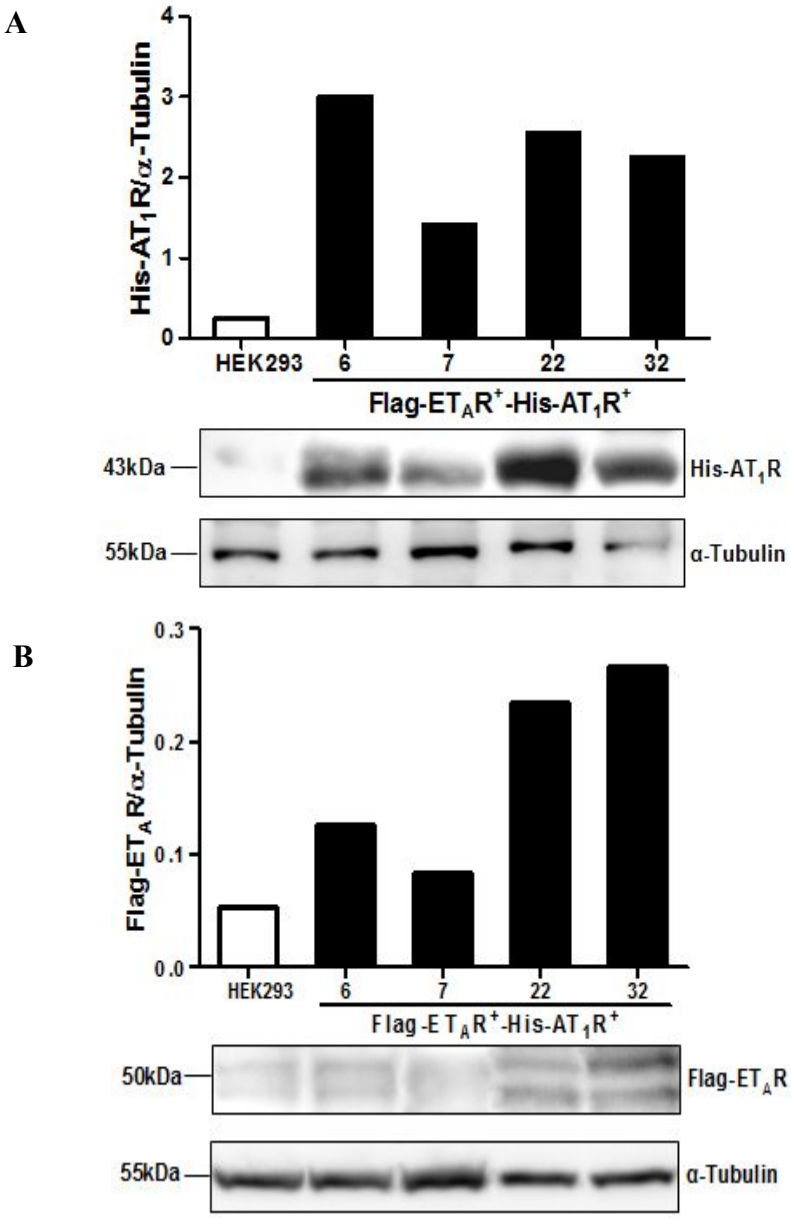


Figure 26. Tagged receptor expression in Flag-ET_AR^+-His-AT_1R^+ cell clones. Cells were grown in the presence of G418 and Blasticidin S. Total cell extracts were analyzed by western blot with anti-His (A) and anti-Flag (B) antibodies. α-Tubulin was used as loading control.

4.1.3.2 Activation of the tagged receptors in response to endogenous stimulation

The effects of Ang II and ET-1 as endogenous ligands of the receptors on the activation of ERK1/2 were studied in the Flag-ET_AR^+-His-AT_1R^+ cell clones. The specificity of the activation of the receptors by their natural ligands was verified by using AT_1R and ET_AR blockers, Valsartan and BQ123 respectively. In Figure 27A, there was an elevation of phosphorylated ERK1/2 after exposure to Ang II compared to the non-transfected cells and the effect could be blocked by Valsartan. As verified by western blot (Figure 27B), there was an increase in pERK1/2 after ET-1 treatment in comparison to the HEK293 cells and the effect can be blocked by pre-incubation with BQ123. These experiments showed that the activation leading to ERK

phosphorylation was specific to the tagged receptors. The highest increase of ERK activation induced by Ang II was observed in clone 32 and the highest increase of ERK activation induced by ET-1 was observed in clone 6. Therefore, these two clones were used in further experiments.

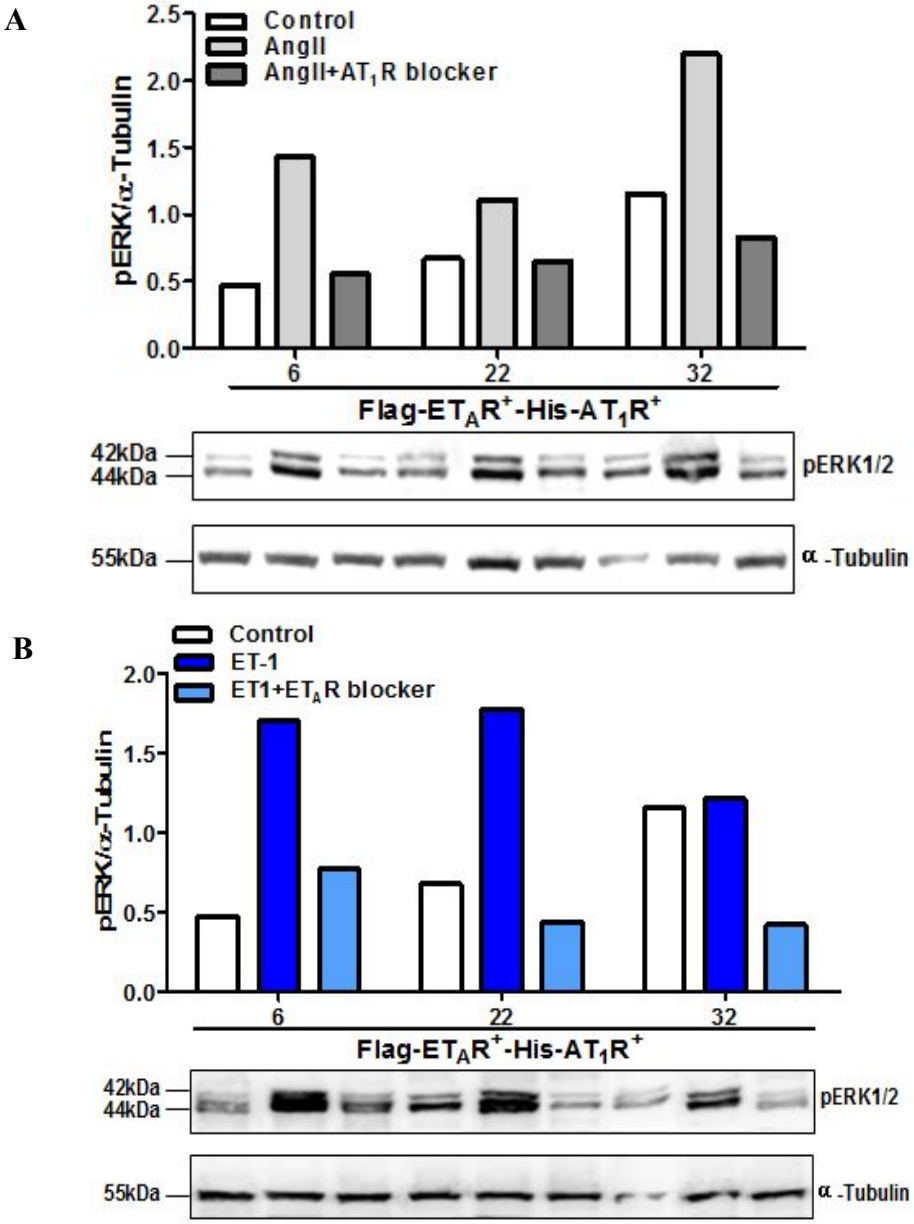


Figure 27. Activation of the tagged receptors in response to endogenous stimulation, Ang II (A) and ET-1 (B). Cultured cells were starved for 24 h in serum-free medium. Then cells were preincubated for 1h with AT_1R or ET_AR blocker (100 mM), and treated with Ang II (10 mM) or ET-1 (100 mM ） for 10 min. Cell extracts were immunoblotted with an anti-pERK antibody to monitor ERK activation. α-Tubulin was used as loading control. Non-stimulated cells were used as control.

4.1.3.3 Activation of the tagged receptors in response to immune stimulation

As observed in the His-AT_1R^+ and Flag-ET_AR^+ cell line in response to AT_1R-Abs and ET_AR-Abs, ERK1/2 phosphorylation was highly induced in the Flag-ET_AR^+-His-AT_1R^+ cell lines compared to non-stimulated cells (Figure 28).

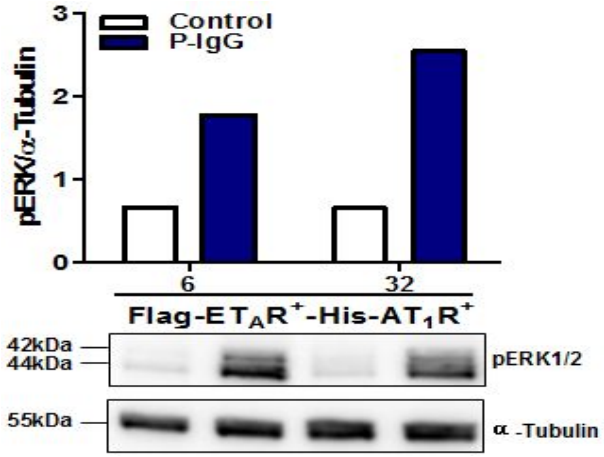


Figure 28. Activation of the tagged two receptors in response to immune stimulation in Flag-ET_AR^+-His-AT_1R^+ cell lines compared to non-stimulated cells. Cultured cells were starved for 24 h in serum-free medium, and then cells were stimulated with P-IgG (1.5 mg/mL) or control-IgG for 1h. Cell extracts were analyzed by western blot with an antibody against pERK1/2. α-Tubulin was used as loading control. Non-stimulated cells were used as control.

To validate that the effects of patient IgG (P-IgG) came from the binding of the antibodies to the receptors, $F(ab')_2$ fragments without Fc fragments were isolated. $F(ab')_2$ fragments induced activation of ERK1/2 which was examined by western blot. As shown in Figure 29, both $F(ab')_2$ fragment and P-IgG increased ERK1/2 phosphorylation in the dual receptors cell lines (data of the second dual cell line not shown) but $F(ab')_2$ was significantly more biologically active than P-IgG ($P<0.05$). Moreover, non-transfected cells showed less activation than receptors over-expressing cells (Figure 29). The results showed that $F(ab')_2$ fragments not only retained the original specificity of anti-AT_1R and ET_AR antibodies but also enhanced the biological function of the autoantibodies.

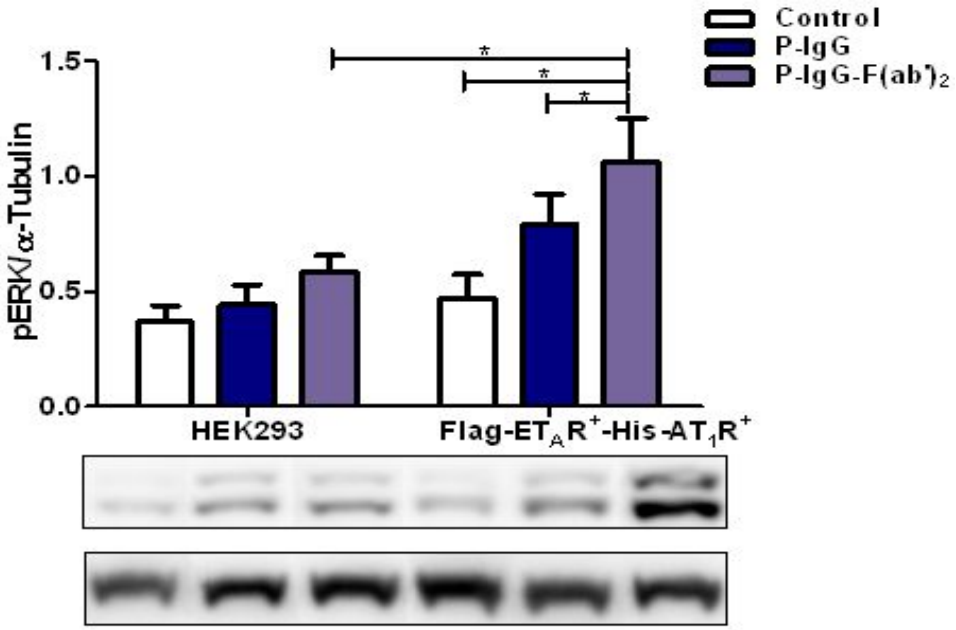


Figure 29. $F(ab')_2$ from P-IgG increased activation of ERK1/2 in the Flag-ET_AR^+-His-AT_1R^+ cell line compared to stimulation with P-IgG. Cells were stimulated with P- IgG or the corresponding $F(ab')_2$ for 10 min. Cell extracts were analyzed by western blot with an antibody against pERK1/2. α-Tubulin was used as loading control. Non-

stimulated cells were used as control. HEK293 were used as negative control. Three experiments were performed. **P <0.05*.

4.2 Interaction between Angiotensin II type 1 and Endothelin-1 type A receptors

Clinical studies have found that the AT_1R-Abs and ET_AR-Abs were detected simultaneously in many auto-immune mediated diseases. This prompted us to do further experiments using the new tools described previously.

Anti-Flag antibody can immuno-precipitate Flag tagged ET_AR protein in the Flag-ET_AR^+ stable cell line (Figure 30A) but the immune-precipitation of His-AT_1R in the His-AT_1R^+ stable cell line with the anti-His antibody was not successful (Figure 30B). Thus, the anti-Flag and anti-ET_AR antibodies were used to perform the co-immunoprecipitation experiments.

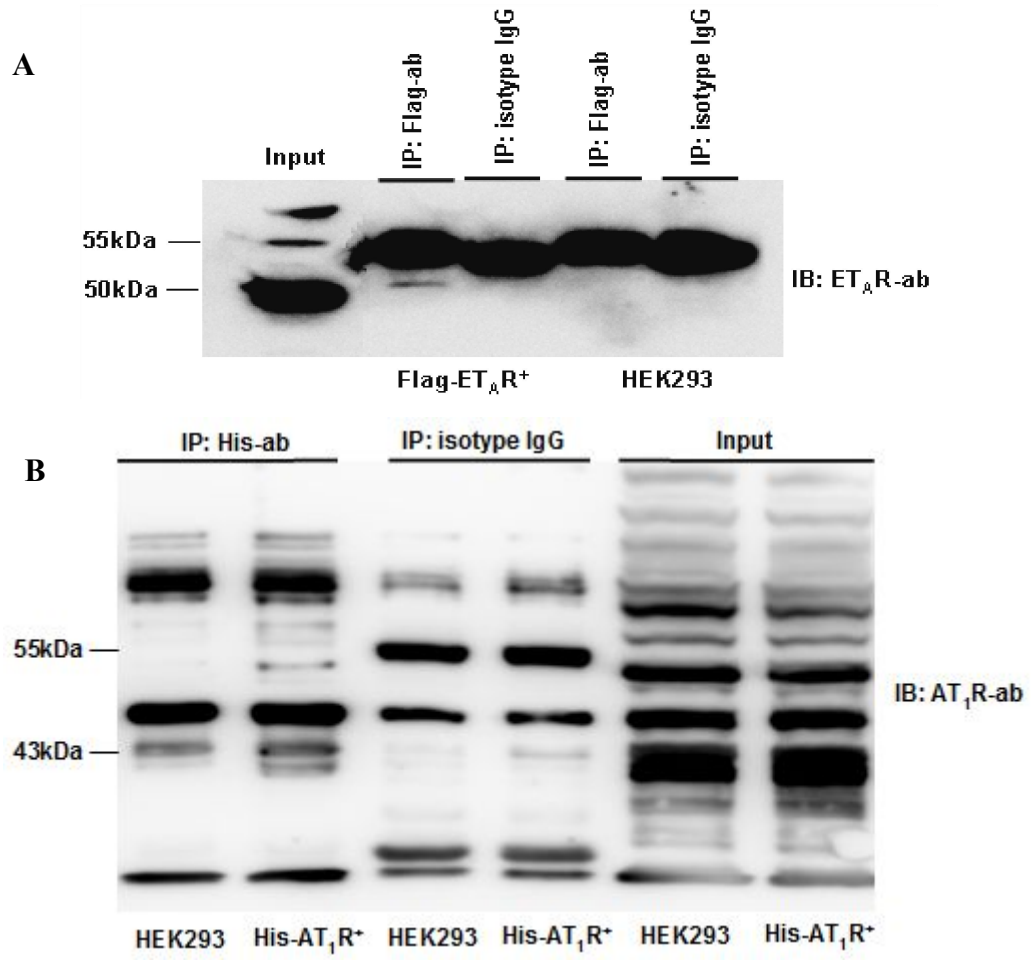


Figure 30. Immunoprecipitation of Flag-ET_AR and His-AT_1R protein in overexpressing cell lines. A. Flag-ET_AR protein in cell extracts of Flag-ET_AR^+ cell lines was immuno-precipitated by an anti-Flag antibody, followed by immunoblotting (IB) with an anti-ET_AR antibody. B. His-AT_1R in cell extracts of the His-AT_1R^+ cell line were immuno-precipitated by an anti-His antibody, followed by IB with an anti-AT_1R antibody. Normal isotype IgG was used as a control for IP. HEK293 was used as a negative control.

Western blot analysis has proved that Flag-ET_AR and His-AT_1R were expressed higher in the dual receptor's stable clone 32 than in clone 7 (Figure 26). Immuno-precipitation experiments showed that the anti-Flag antibody could recognize Flag tagged ET_AR in the Flag-ET_AR^+-His-AT_1R^+ cell clones (clone 7 and 32), and that Flag-tagged ET_AR was more detected in clone 32 than in clone 7 (Figure 31).

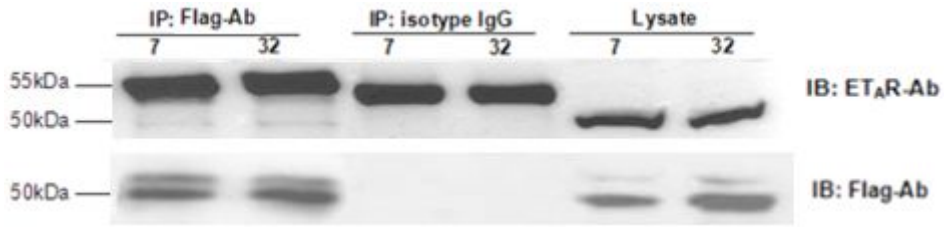


Figure 31. Flag-ET_AR protein in cell extracts of Flag-ET_AR^+-His-AT_1R^+ cell clones (clones 7 and 32) was immunoprecipitated by an anti-Flag antibody, followed by IB with anti-ET_AR and anti-Flag antibodies. Normal isotype IgG was used as a control for immunoprecipitation.

Then co-immunoprecipitation experiments were performed to establish whether there is a direct interaction between Flag tagged ET_AR protein and His tagged AT_1R protein. The analysis revealed that AT_1R protein might bind to Flag-ET_AR in both clone 7 and 32 (Figure 32).

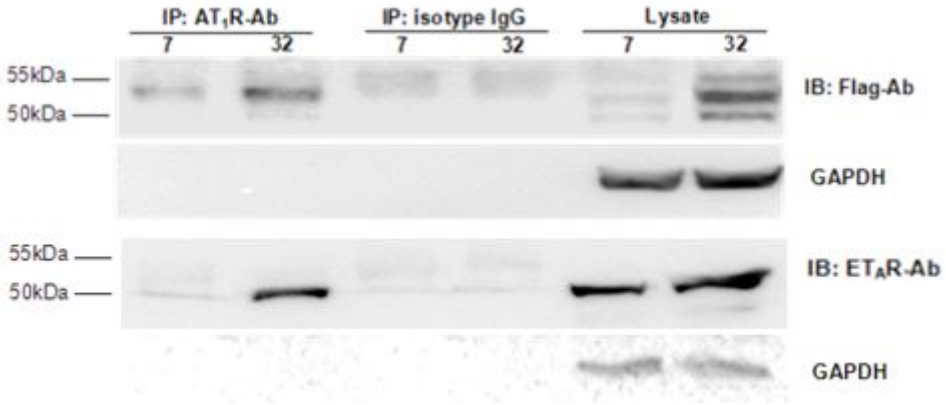


Figure 32. Co-immunoprecipitation of His-AT_1R with Flag-ET_AR in Flag-ET_AR^+-His-AT_1R^+ cell clones (7 and 32). Cell extracts from clones 7 and 32 were immuno-precipitated by an anti-AT_1R antibody, followed by IB with anti-ET_AR and anti-Flag antibodies. Normal isotype IgG was used as a control for IP.

4.3 Study of the effects of immune stimulation on the structure of the receptors

Little is known about the binding of auto-antibodies on AT_1R but in the context of preeclampsia and kidney graft rejection, the second extracellular of the receptor seemed to play a key role[96]. As for the ET_AR, the exact role played by ECL2 in its activation upon immune stimulation has yet to be discovered. In order to study the effects of immune stimulation on the ECL2 of both receptors, a GPCR activation assay was generated, using a yeast model which has already been successfully used on the A_{2B} receptor[97].

4.3.1 Expression of the human AT_1 and ET_A receptors in the GPCR activation assay

We obtained the MMY yeast system from GlaxoSmithKline. The Saccharomyces cerevisiae strain had been modified genetically to serve as a reporter system with growth as an output parameter. This system is an ideal background for studying the activation of an individual GPCR, since its only endogenous GPCR was removed while still maintaining the complete GPCR-signaling mechanism. Several studies have shown that the system is predictive of the mammalian situation. Different MMY strains were tested to find the best to express AT_1R and ET_AR and study their response to their endogenous ligands, Ang II and ET-1 respectively. For each receptor a strain was found in which the receptors reacted to its ligand in a dose-dependent manner (figure 33).

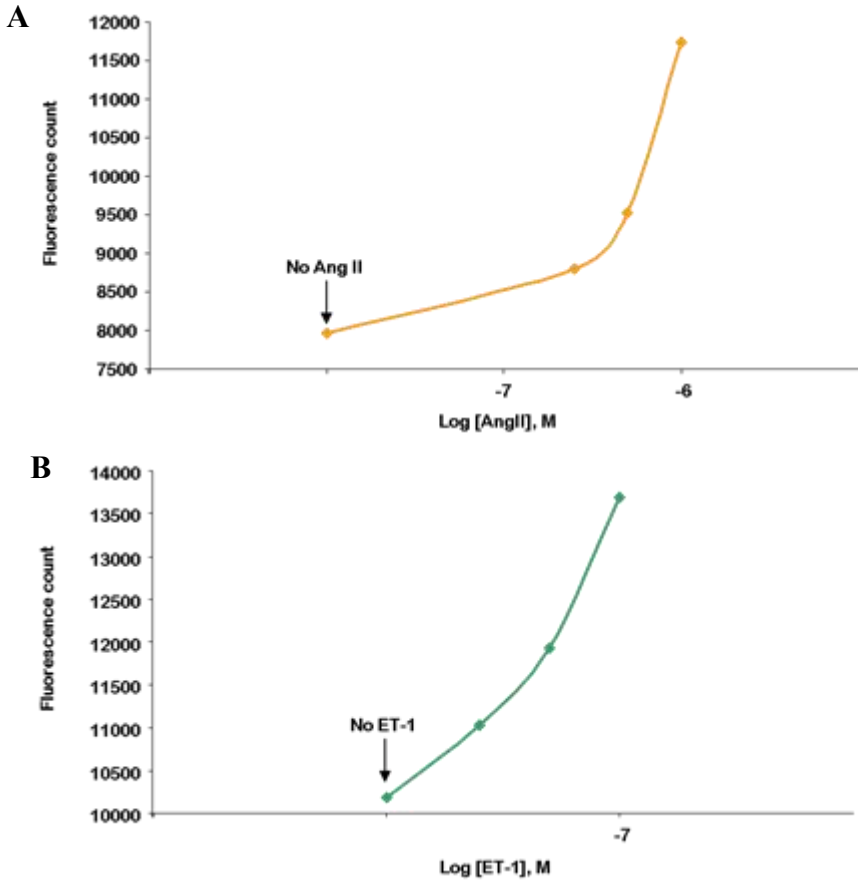


Figure 33. Activation of AT_1R and ET_AR by their endogenous ligands. A. Ang II dose-dependent activation of AT_1R in the MMY14 strain. B. ET-1 stimulates ET_AR in a dose-dependent manner in the MMY12 strain. The first measure gives the yeast growth without ligand.

4.3.2 Generation of ECL2 swapped receptors

Human cDNAs coding for either AT_1R or ET_AR were cloned in a plasmid enabling their expression in the MMY yeast system. A protein alignment was generated to compare the amino acid sequences of the two receptors, in particular in the second extracellular loop. The protein

sequence of ECL2 of ET_AR contains 31 amino acids (AA) from positions 222 to 252, ECL2 of AT_1R contains 28 AA from position 198 to 225 (Figure 34).

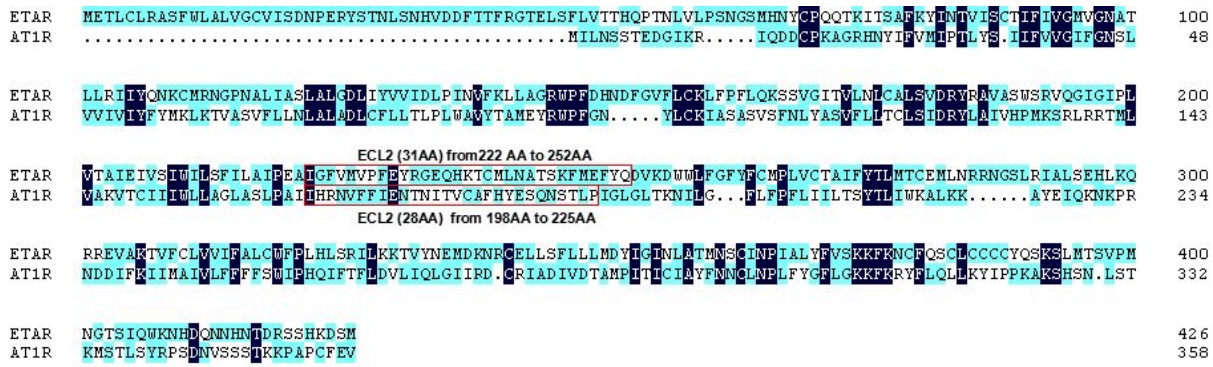
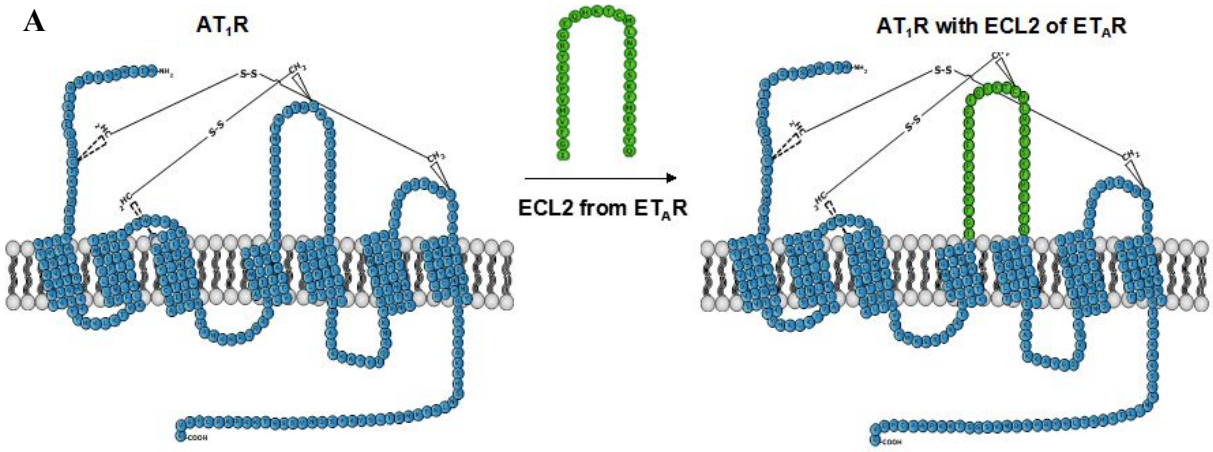


Figure 34. The sequence alignments of ET_AR and AT_1R protein sequences generated by Clustal X2. In the sequences, red frame indicates the ECL2 of the receptors; the dark blue color indicates an highly conserved residues; cyan blue indicates poorly conserved residues.

In order to see whether the antibodies found in patients bind to the second extracellular loops of the receptors and if these bindings are specific to the amino acid sequence, we decided to swap the ECL2 of AT_1R and ET_AR. Site-directed mutagenesis was performed to integrate the ECL2 of ET_AR in AT_1R (Figure 35A) and the ECL2 of AT_1R in ET_AR (Figure 35B). After PCR amplification with primers designed for site-directed mutagenesis, the amplified mutated sequences were separated by gel electrophoresis (Figure 36). After treatment with the Q5® Site-Directed Mutagenesis Kit and transformation, five clones were isolated and sequenced to identify successfully ECL2 swapped receptors mutants.



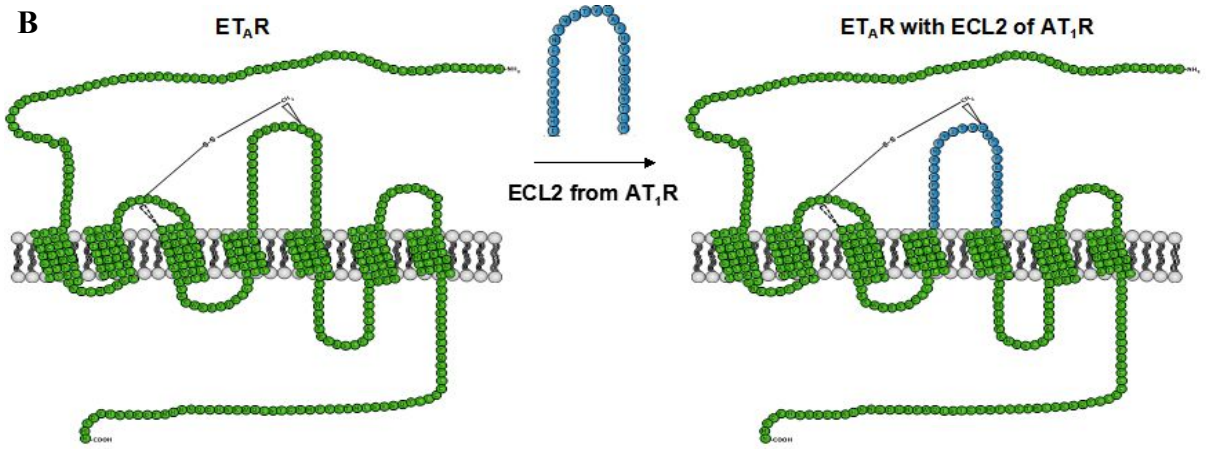


Figure 35. Secondary structure model of ECL2 swapped receptors. A. The loop-swapped model of AT_1R. ECL2 of AT_1R was substituted by the ECL2 from ET_AR. B. The loop-swapped model of ET_AR. ECL2 of ET_AR was substituted by the ECL2 from AT_1R.

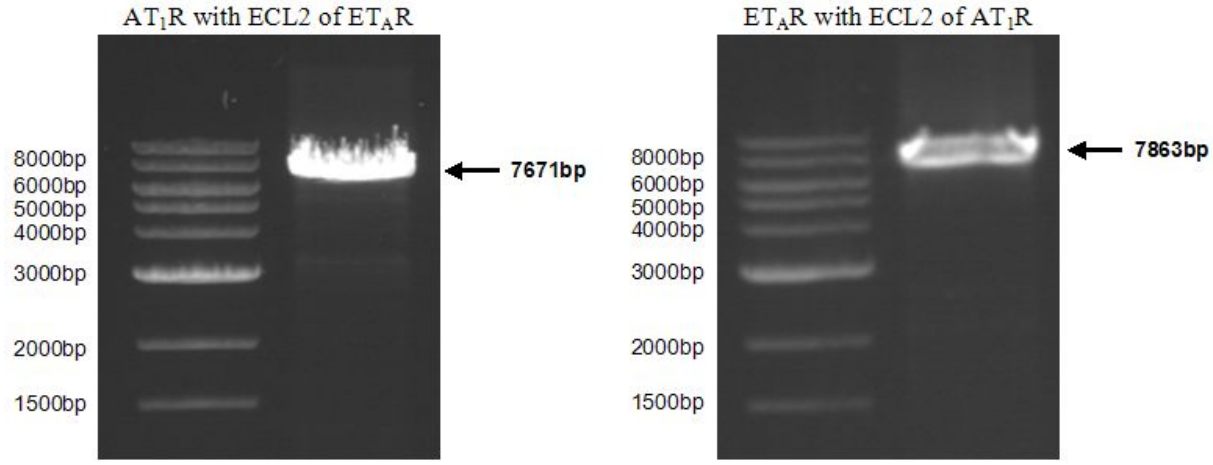


Figure 36. Amplified mutated plasmids separated by gel electrophoresis. The AT_1R with ECL2 of ET_AR plasmid and ET_AR with ECL2 of AT_1R plasmid were 7671 bp and 7873 bp long respectively

4.3.3 Effects of immune stimulation on the ECL2 swapped receptor mutants

In order to test the effect of the ECL2 swapped receptors, endogenous and immune stimuli were used to stimulate yeasts transformed with the mutant plasmids. Growth was monitored and compared to the one of yeasts transformed with the wild-type receptor. In contrast to the absence of stimulation, Ang II (Figure 37A) and patient IgG (Figure 37B) activate significantly and dose-dependently wild type AT_1R; whereas AT_1R with the ECL2 of ET_AR responded significantly less to both stimulations. Concerning ET_AR, in contrast with the non-stimulated yeasts, ET-1 (Figure 38A) and patient IgG (Figure 38B) activated both wild type and ET_AR with the ECL2 of AT_1R significantly and dose-dependently. These results showed that endogenous and immune-mediated activation of the receptors depends on the second extracellular loop in AT_1R but not in ET_AR.

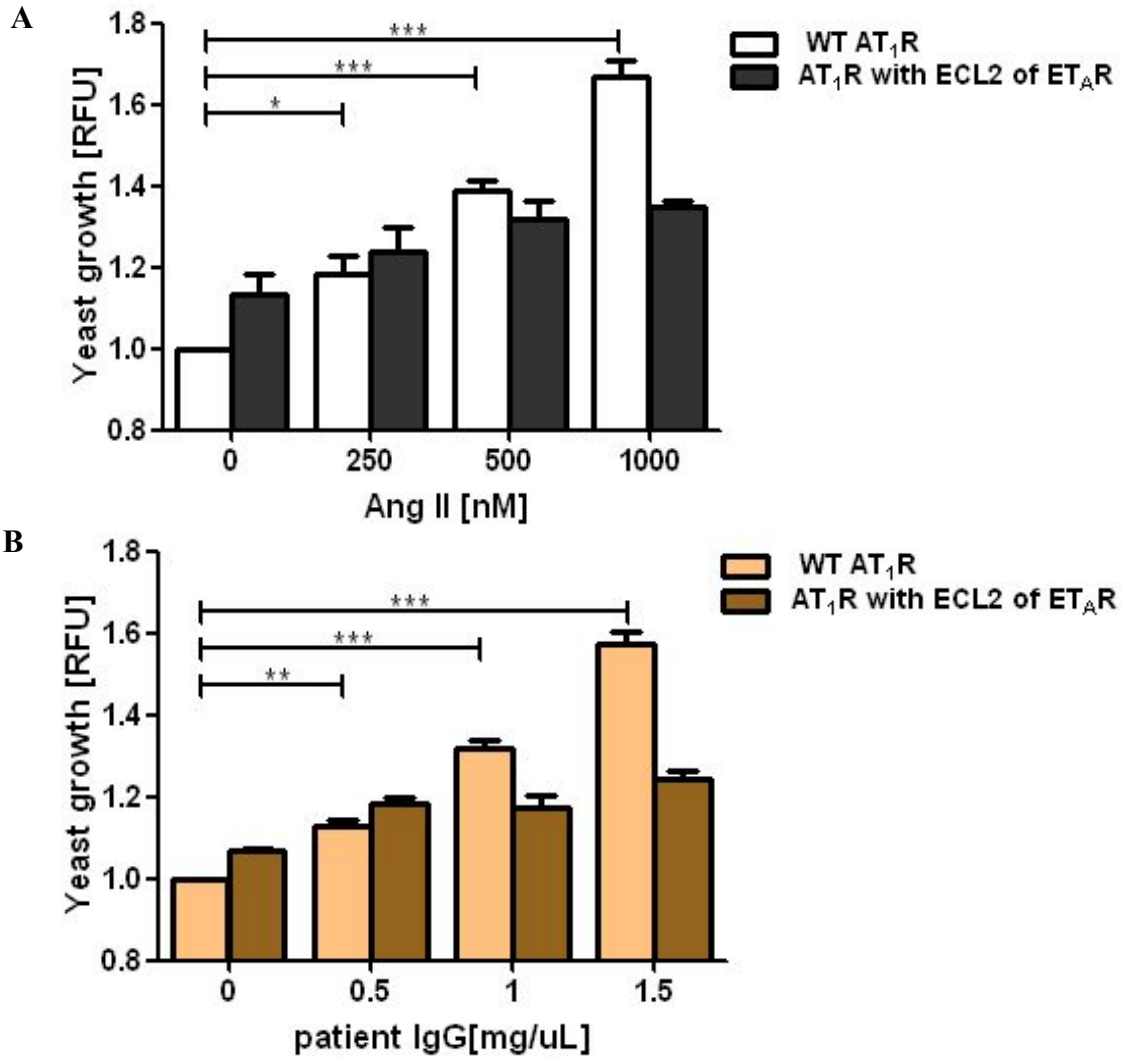
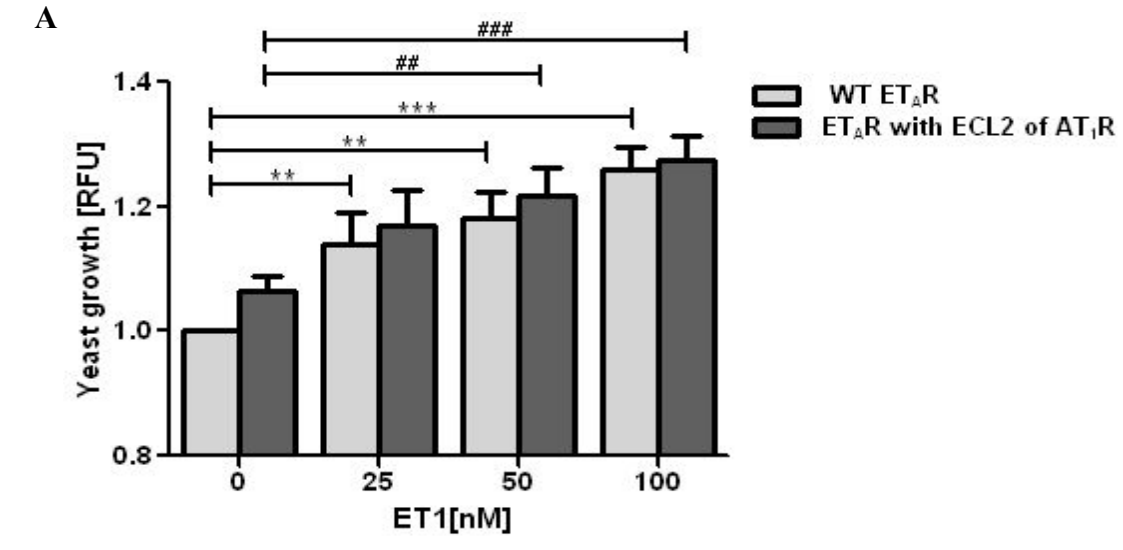


Figure 37. Endogenous and immune-mediated wild type and mutant AT_1R activation. A. Activation of AT_1R with ECL2 of ET_AR and wild type AT_1R induced with different concentrations of Ang II. B. Activation of AT_1R with ECL2 of ET_AR and wild type AT_1R induced with different concentrations of P-IgG. 0 mM and 0 mg/μL were used as negative control. Experiments were repeated at least three times. Compared with wild type receptor without stimulation, *$P<0.05$, **$P<0.01$, ***$P<0.001$.



B

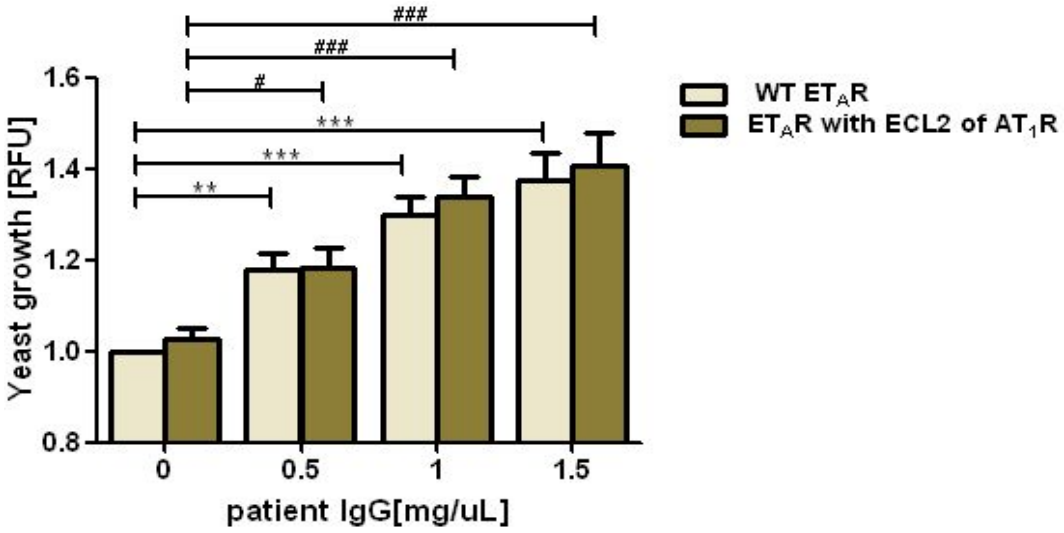


Figure 38. Endogenous and immune-mediated wild type and mutant ET_AR activation. A. Activation of ET_AR with ECL2 of AT_1R and wild type ET_AR induced with different concentrations of ET-1. B. Activation of ET_AR with ECL2 of AT_1R and wild type ET_AR induced with different concentrations of P-IgG. 0 mM and 0 mg/μL were used as negative control. Experiments were repeated three times. Compared with wild type receptor without stimulation, $^{**}P<0.01$, $^{***}P<0.001$. Compared with mutant receptor without stimulation, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$.

5 Discussion

Increasing evidence suggests a role of antibodies directed against non-HLA antigens, like Angiotensin II type 1 and Endothelin-1 type A receptors, in the pathogenesis of obliterative vasculopathy. In this work, tagged AT_1R or/and ET_AR over-expressed stable cell lines were generated. The functionality of both receptors was verified with their natural ligands, Ang II and ET1 respectively, and their response to autoimmune antibodies was monitored via ERK activation. Moreover, the stable cell lines were used to show that the $F(ab')_2$ from patients IgG are biologically active. The Flag-ET_AR^+-His-AT_1R^+ stable cell line was used as a tool for studying the interaction between AT_1R and ET_AR. The co-IP results showed that both anti-Flag and anti-ET_AR antibodies can recognize the AT_1R protein, which suggested that ET_AR and AT_1R may form receptor heteromers in recombinant systems. To get a more comprehensive understanding of the effects of immune stimulation on the structure of the receptors, a GPCR activation assay was generated. Ang II- and patient IgG-induced AT_1R activation depended on the second extracellular loop while the activation of ET_AR induced by AT_1R-Abs and ET_AR-Abs was independent on the ECL2.

5.1 Generation of cell lines expressing Angiotensin II type 1 and Endothelin-1 type A receptors individually or dually

Stable cell lines expressing AT_1R and ET_AR individually or dually were generated in HEK293 and the expression level and functionality of the receptors was verified. Affinity tags have become powerful tools for basic biological research which can be widely used to facilitate the purification and detection of proteins of interest, as well as the separation of protein complexes[98]. In this study, the human AT_1R sequence which consists of 359 amino acids was successfully cloned into the pSELECT-NHis-blasti vector in frame with 6× His epitope (His-AT_1R). The anti-His antibody could recognize the His tagged AT_1R protein by western blot (Figure 16). Because of the important role of AT_1R in physiology and pathophysiology, many investigations generated AT_1R recombinant plasmid for understanding molecular mechanisms of AT_1R in health and disease[99]. In those studies, different tags were used to produce AT_1R recombinant proteins. For example, Wennmann et al.[100] constructed the N-terminal 3× Flag-tagged human AT_1R, cloned it into the retroviral expression vector pQCXIP and established a stable AT_1R-overexpressing human podocyte cell line. Some other recombinant AT_1R plasmid-encoding rat $AT_{1A}R$ have emerged in researches, such as an expression vector encoding rat $AT_{1A}R$ fused with hemagglutinin (HA) tag for studying the differential β-arrestin binding of AT_1 and AT_2

angiotensin receptors[99,101]. One reason why a different tag was chosen for generating the AT_1R recombinant protein is because we already had a Flag-ET_AR plasmid. Another reason is that the His-tag is one of the most frequently used affinity tags for protein enrichment[98]. Until now, there are not so many accurate researches about ET_AR protein with a tag *in vitro*. When Gregan et al.[73,72] studied the hetero- and homodimerisation of Endothelin A and Endothelin B receptors, they mentioned that HEK293 cells were stably transfected with plasmids encoding fusion proteins comprising an N-terminal c-myc epitope-tagged ET_AR. In this study, it was observed that the anti-Flag antibody can distinguish the fusion Flag-ET_AR protein in transient transfected HEK293 cells (Figure 21).

Another key factor for making stable cell line is the choice of the cells. In the literature, Human embryonic kidney cells (HEK293), African Green Monkey kidney cells (COS-7) and Chinese hamster ovary cells (CHO) are the most popular cell lines, especially for transfection experiments with recombinant plasmids[102,103]. However, COS-7 cells contain the SV40 large T antigen which allowed them replicate plasmids containing the SV40 origin and most mammalian expression vectors contain SV40 origin[104]. The actual replication of the plasmids is presumably so high that cells will eventually die. This makes COS-7 cells very suitable for transient but not for stable transfection. HEK293 and CHO cells not containing SV40 large T antigen are easy to transfect and readily form colonies when stably transfected and selected.

Thomas et al.[105] concluded in a review that HEK cell line has been extensively used as an expression tool for recombinant proteins and has also seen widely used in stably transfected forms to study a variety of cell-biological. Accordingly, HEK293 were employed to make stable transfection for expressing His-AT_1R or/and Flag-ET_AR proteins. The results demonstrated that the three stable cell lines generated could over-express the receptors either in total cell extracts or in cell membrane fractions.

5.2 Biological effects of AT_1R-Abs and ET_AR-Abs in these cell lines

The expression of AT_1R and ET_AR has been found in several cell types, both receptors mediate the activation of various pathways, depending on the function of the relevant tissue[44]. Although Ang II binds to both AT_1R and AT_2R, the majority of Ang II actions are mediated via AT_1R[106]. ET_AR binds endothelins and has a higher affinity for ET1, as compared to ET_BR[107]. It has been confirmed that the natural ligands of AT_1R and ET_AR are Ang II and ET1, which can initiate canonical signaling by means of activating of ERK 1/2 cascade *in vitro*[108]. In this study, the cell lines were checked not only by detecting the receptor quantity but also by verifying the functionality of both receptors.

Agonistic autoantibodies against the AT_1R and the ET_AR have been identified in patients with SSc[12,44,109] and transplant allograft vasculopathy[11,82]. Dragun et al.[10] reported that *in vitro* stimulation of vascular cells with AT_1R-Abs from renal-allograft rejection induced phosphorylation of ERK 1/2. Riemekasten et al.[12] revealed that both autoantibodies exerted biological effects, since they induced ERK1/2 phosphorylation and increased TGFβ gene expression in endothelial cells that could be blocked with specific receptor antagonists. In this study, it was also demonstrated that autoantibodies activated ERK 1/2 phosphorylation in the normal HEK cells, and confirmed that they are biologically active in that they induce receptor-directed ERK1/2 phosphorylation in the cell line over-expressing AT_1R or/and ET_AR (Figures 20,26, 30).

Antibody fragmentation is accomplished using reducing agents and proteases that digest or cleave certain portions of the immunoglobulin protein structure[110]. It is useful to study or make use of the activity of one portion of an immunoglobulin without interference from other portions of the molecule[111]. In early 1977 when studying the effect of rheumatoid factor (RF) in early rheumatoid arthritis, Carson et al.[112] exploited isolated human IgG-Fc and $F(ab')_2$ fragments to avoid false-positive results and demonstrated that the Fc fragment cannot bind antigen and that it was responsible for the effector functions of antibodies, such as complement fixation. As fragment antibodies do not have Fc portions, they would not be interfered by Fc-mediated effector functions[111]. Thus, the removal of the Fc segment has been reported reducing the nonspecific binding of the antibody because many cells have receptors that bind the Fc region leading to Fc interactions[113]. Fragmentation of antibodies results in altered physiochemical features of these molecules[114]. $F(ab')_2$ fragments are smaller than whole IgG molecules and have two variable domains binding the epitope on its specific antigens[111]. Lacking Fc regions, $F(ab')_2$ fragments do not retain the complement-binding function or reduced interaction with non-specific proteins[115]. In this study, $F(ab')_2$ fragments not only retained the original specificity of total patient IgG but also enhanced the biological activity of autoantibodies (Figure 30). Thus, AT_1R and ET_AR antibodies play an important role in transducing intracellular signals by binding to receptors on the surface of cells. $F(ab')_2$ fragments as a functional fragment with more biologically activity could be widely used in basic studies for obtaining reliable findings on better defining autoantibodies independent activation mechanism in auto-immune diseases. Further studies to determine the influence of $F(ab')_2$ fragments of the two autoantibodies on our cell model are required.

5.3 Putative interaction between Angiotensin II type 1 and Endothelin-1 type A receptors

Recently it has been well documented that AT_1R and ET_AR have an important clinical significance in transplantation[11,82,116] and SSc[12,44,28,109]. The AT_1R-Abs and ET_AR-Abs are simultaneously involved in the pathogenesis of obliterative vasculopathy. Cross-talk between AT_1R and ET_AR has been described in some studies. For example, AT_1R-Abs-induced hypertension in pregnant rats was attenuated by either oral administration of the AT_1R antagonist losartan or an ET_AR antagonist[117] and the increase in ET-1 transcript in response to AT_1R-Abs-induced hypertension was abolished by administration of an AT_1R antagonist.[118] Lin et al.[119] reported that Ang II enhanced ET-1-induced vasoconstriction by up-regulating ET_AR expression and ET-1/ET_AR binding, suggesting the synergistic effect of Ang II and ET1 on the pathogenic development of hypertension. Becker et al.[28] suggested that autoantibody-mediated receptor cross-talk, together with enhanced reactivity to natural ligands, could represent an additional pathophysiological aspect of vascular dysregulation. All this evidence either from human or animal models indicates that ET_AR and AT_1R may interact with each other functionally, nevertheless little is known about physical interaction between these two receptors. The simultaneous occurrence of anti-AT_1R and anti-ET_AR Abs implies the importance of possible receptor heterodimerization, which could affect the influence of agonists and antagonists on signal transduction[109]. Therefore, in the present study, it was investigated for the first time whether AT_1R physically interact with ET_AR to form heterodimers.

There is increasing evidence that different GPCRs may form heterodimers and this can affect the function of each agonist, resulting in significant functional interactions[120]. Generally, GPCRs heterodimer are difficult to analyze in native cells and tissues[121]. It has also been suggested that some examples of GPCR dimerization may, at least in part, be artifacts derived from the high levels of expression that can often be achieved in heterologous cell systems[121]. Thousands of GPCR research studies have utilized heterologous expression systems such as HEK293[122]. HEK cell line co-expressing two different receptors is usually a useful tool for studying the interaction between two membrane receptors[105]. Many studies generated different receptor expression tools depending on their aims. For example, HEK cells co-expressing myc-ET_A and Flag-ETB stably were performed to investigate ET-receptor dimerization[112,113]; cells co-expressing AT_1R and B_2R[59] or HA-CB_1R[123] were generated for investigating the interaction between AT_1R and B_2R or AT_1R and CB_1R.

In this study, co-IP experiments were carried out to observe the AT_1R and ET_AR heterodimerization. The results first proved that anti-Flag antibody can precipitate Flag-ET_AR protein either in cells over-expressing ET_AR or in double receptor stable cell lines and subsequently showed that anti-Flag and anti-ET_AR antibodies can recognize the His-AT_1R protein in the double receptor stable cell lines. To verify this, the Flag-ET_AR protein should be recognized by anti-His and anti-AT_1R antibodies, which could not be achieved. This was a limitation of this study, different antibodies directed against AT_1R were tested and none of them was specific. In 2013, Herrera et al.[99] and Elliott et al.[124] studied different AT_1R antibodies, including the ones we used, and reported that there is no specificity of commercial antibodies for detecting AT_1R protein in experimental systems. Considering that the AT_1R protein in the expression system we designed was a recombinant protein, His-tagged AT_1R, the anti-His antibody could have been used. Unfortunately, the anti-His antibody could be effectively-used in western blot for detecting His-AT_1R, but it was unspecific in co-IP experiments. In their study for testing the ability of the commercial antibodies to detect AT_1R, Herrera et al.[99] used the same anti-His antibody as ours for the exogenous His-tagged proteins by western blot, and the anti-His antibody detected multiple bands close to 39 kDa in cells transfected with His-AT_1R plasmid. In 2006, Debeljak et al.[125] published an article about variability in the immunodetection of His-tagged recombinant proteins and presented several factors that could be disturbing the detection of His-tagged recombinant proteins. Although some technical problem existed, AT_1R and ET_AR were co-immunoprecipitated in the dual stable cell line, reinforcing the hypothesis that the corresponding receptors form heterodimers in native cells or tissues.

Satake et al.[126] mentioned in a review that interaction between GPCRs should be proved by at least two different experimental procedures including co-IP experiments, fluorescence resonance energy transfer (FRET), or bioluminescence resonance energy transfer (BRET). So our further experiments will be continued using FRET or BRET and both receptors' cDNA will be cloned in new plasmids upstream of a fluorochrome.

To explain the functional interaction between the two receptors, it was speculated that the two receptors form stable heterodimers, resulting in enhanced activation of AT_1R and ET_AR signaling. It was possible to achieve dual receptor inhibition of AT_1R and ET_AR signaling using only a single receptor antagonist (Figure 27). The explanation might be that both of AT_1R and ET_AR are vasoconstrictors[51] and AT_1R has a synergistical effect, where ET_AR signaling can cause vasoconstriction and lead to hypertension and vascular endothelium injury[127]. From a medical perspective, AT_1R and ET_AR interaction would be of outstanding value because both of these two receptors have the same adverse effect on diseases. Rozenfeld et al.[123] suggested that

dimerization and potentiation of AT_1R signaling may be a universal phenomenon that occurs as a natural consequence of simultaneous expression of the two receptors. Thus, the results demonstrated that AT_1R and ET_AR functionally interacted, resulting in the potentiation of AT_1R, and ET_AR signaling activated by autoantibodies proved the inference.

5.4 Impact of the second extracellular loop on immune stimulation

In this study, using a GPCR activation assay based on a yeast model, it was shown that receptors reacted to their endogenous ligands and to autoantibodies in a conspicuous dose-dependent manner. It was also demonstrated that the second extracellular loop of AT_1R plays a role in the immune activation of the receptor and that this is not the case in ET_AR. Woolley et al.[128] suggested that the extracellular loops of GPCRs which are important for receptor function, contribute to protein folding, provide structure to the extracellular region and mediate movement of the transmembrane helices on activation. Feng et al.[129] reported that ECL2 of AT_1R directly interacts with the Ang II. The results made it clearer that Ang II can bind to the ECL2 of AT_1R directly even without considering the crystal structure of the AT_1R. This loop is targeted by autoantibodies that activate AT_1R in several pathologies such as preeclampsia[86], malignant hypertension and vascular allograft rejection[10]. Dragun et al.[10] identified that AT_1R-Abs bound to two different epitopes on the ECL2 of the AT_1R. Thus, in accordance with this evidence, we demonstrated that the autoantibodies exerting their immune stimulation on AT_1R depend on the ECL2 of AT_1R. ECL2 is of significance for ligand binding and receptor activation[130]. For instance, it has been reported that ECL2 of the glucagon-like peptide 1 (GLP-1) receptor plays an important role in directing coupling towards stimulation of ERK1/2 activation versus Gs and activation of adenylate cyclase[128]. Mutagenesis studies also confirmed that the ECL2 region is responsible for the receptor subtype selectivity of signaling molecules and its alteration may transform an antagonist to act as an agonist[131,132].

However, regarding the ET_AR, the exact role played by ECL2 in its activation upon ET1 and autoantibodies stimulation has not yet been discovered[77]. This study based on the secondary structure of ET_AR showed that ET1 and autoantibodies can still induce ECL2 mutant ET_AR activation. However, it cannot be affirmed that ET1 and autoantibodies' binding sites in the ET_AR were not on the ECL2 region. A realistic GPCR structure is a three-dimensional structure, and disulfide bridges play an important role in receptor's conformation and the functionality[133]. In 1995, Perlman et al.[134] reported that disulfide bond between conserved extracellular cysteines (Cys-98 and Cys-179) in the thyrotropin-releasing hormone receptor (TRHR) was critical for binding. In 1997, Cook et al.[135] also proved disulfide bonding between extracellular cysteine

residues contributes to the structural integrity of the gonadotropin-releasing hormone receptor (GnRH-R). One explanation of the results is that the ECL2 mutagenesis disabled the disulfide bridge (Cys-158 and Cys-239)[136] between ECL1 and ECL2 of ET_AR which might interfere. Therefore, in further studies, the other extracellular domains of the receptor will be analyzed and their activation tested in response to ET-1 and immune stimulation in order to specify their role.

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Affidavit

"I, Nan, Zhu certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Development of a molecular toolbox to study the cross-talk between Angiotensin II type 1 and Endothelin-1 type A receptors in the context of obliterative vasculopathy] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Curriculum Vitae

My curriculum vitae is not published for privacy reasons in the electronic version of my thesis.

Publications

- Wang X, Zhou Y, **Zhu N**, Wang L, Gu LJ, Yuan WJ. The deposition of Notch1 in hepatitis B virus-associated nephropathy and its role in hepatitis B virus X protein-induced epithelial-mesenchymal transdifferentiation and immunity disorder in renal tubular epithelial cells. J Viral Hepat. 2014 Oct;21(10):734-43.
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- Zhou Y, Yuan WJ, **Zhu N**. The efficacy of anti-viral therapy on hepatitis B virus-associated glomerulonephritis: A systematic review and meta-analysis. Ann Hepatol. 2011Apr-Jun; 10(2):165-73.
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Abstract

- **Zhu N**, Philippe A, Catar R, Riemekasten G, Dragun D. Fragments of AT_1R and ET_AR-antibodies induce complement-independent endothelial activation. Abstract. Transpl Int. 2014 Oct;27 Suppl 3:43.

Poster Presentation

- $F(ab')_2$ fragments of AT1R and ETAR-Abs mediate complement independent mechanisms of endothelial activation

(2014 - 6. Jahrestagung der Deutschen Gesellschaft für Nephrologie (DGfN) - Berlin)

- Fragments of AT1R and ETAR-Antibodies induce complement-independent endothelial activation

(2014 - 23. Jahrestagung der Deutschen Transplantationsgesellschaft (DTG)-Mannheim)

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